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A HIERARCHY OF SELF-LIMITING REACTIONS AS THE BASIS OF CELLULAR DIFFERENTIATION AND GROWTH CONTROL

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New information from the fields of embryology, genetics and microbiology can be added to the older gradient and organizer concepts and synthesized into a general theory of cellular differentiation and embryonic pattern formation.

At the start it is necessary to point out what is believed to be an error in some current theoretical statements. This is the statement that differentiation is irreversible (Weiss, 1950). It can be agreed that a cell of one differentiated type is stabilized and does not, under stable environmental conditions, change its type. The idea of irreversibility seems to arise from the fact that cells may maintain stable types in tissue cultures for many generations. In spite of the fact that some cells remain stable during culture, a wealth of experimental material clearly demonstrates that cell type may change fundamentally during regeneration in vivo.

It has long been known that cellular differentiation involves the production and maintenance of stable types without the absolute loss, in many cases, of the ability to form other cell types. In plants, somatic cells retain a latent ability to produce a whole plant. It appears that even a single somatic cell may be sufficient as a source of a regenerated plant in Thallophyta and Bryophyta as well as in the higher plants. Differentiated somatic cells of a leaf of Begonia, after isolation of the leaf, lose their former characteristics, grow and produce entire plants (Goebel, 1900). More recently it has been demonstrated that parenchymal cells of roots of carrots can also dedifferentiate, grow and transform to various tissues (Buvat, 1941).

In animals there are also well established cases of dedifferentiation and redifferentiation along new pathways. Two of these, Wolffian lens regeneration from the iris and regeneration of visual cells from tapetum nigrum in Amphibia, are particularly striking (Wolff, 1895; Wachs, 1920; Stone, 1950). Both transformations involve loss of pigment and other peculiar features of the differentiated cells followed by growth of the dedifferen-

tiated cells and subsequent development into wholly different cell types, visual rods in one case and lens fibers in the other. Recently evidence has been presented that epidermis transforms to mesenchymal tissues during limb regeneration in Amphibia (Rose, 1948; Hay, 1951).

It seems quite certain that differentiation does not always involve absolute loss of potentiality. Many observations and experiments from many phyla must be neglected if one is to believe that differentiation is irreversible.

The apparent loss of widespread potency during differentiation is a well known phenomenon. For example, as Yamada (1937) has shown, the ventral equatorial region of an amphibian gastrula or early neurula is on its way to forming blood, blood vessels and some mesothelium. If that region is isolated it will in addition produce some lateral tissues, for example, renal tubules. By the test of isolation the ventral mesodermal tissue has gone so far along the path of determination that it cannot produce the dorsal structures, notochord and segmented muscle. These potencies, though suppressed, can be shown to persist and can be realized after treatment with ammonia (Yamada, 1950). An ammonia solution of approximately pH 12 used for several minutes on the isolated ventral zone causes death of some cells, disaggregation of others and a major cytoplasmic change in the living cells expressed as an increase in translucency. This treatment acts as an eraser of the partial differentiation already obtained and yields cells of much wider potency. After treatment, the mesodermal tissues which differentiate most often are not ventral or lateral tissues but are instead dorsal notochord and segmented muscle. Although dorsal tissues are most common in the treated isolates, all tissues may form. This spectrum of differentiation is the same as that attained by isolated dorsal cells without treatment (Holtfreter, 1938; Yamada, 1950). There are other cases in which disruptive treatments increase potency. In an adult frog's limb after amputation there is little differentiation of anything except scar tissue. However, if the stump is treated with NaCl or other deleterious agents the amount of dedifferentiated tissue is increased and the entire spectrum of limb tissues may be realized in the regenerate (Polezhayev, 1936, 1946; Rose, 1944, 1945, 1948).

The situation in the Amphibia may have its counterpart in the differentiation and transformation of antigen types in Paramecia. The fundamental studies of Sonneborn (1948, 1950, 1951) and Beale (1952) concerning transformation of Paramecia from one antigenic type to another provide a model of cellular differentiation. Genetically identical Paramecia can differ from each other by persistent antigenic characteristics of the cytoplasm. As many as eight different cytoplasmic types are known for one genome. Once a Paramecium has differentiated into a particular antigenic type the differentiation may appear to be irreversible. Under standard environmental conditions all of the descendants of an individual of one type will almost certainly be of the same type. This type of inheritance involves cytoplasmic transmission of the differentiated antigens and these, by a mechanism still unknown, prevent the formation of the other seven types of

antigens. That the differentiation is not absolutely irreversible has been demonstrated in two ways. If a particular antigenic type is treated with an antiserum specific for that type and treatment is discontinued shortly before death is expected, the particular antigen is lost and the Paramecium is thereby rendered pluripotent. Which new antigen of the eight known possibilities will appear depends on the environment. The most effective determining factor studied is temperature. Some antigens are more likely to differentiate at low temperature and others at high temperature. Certain temperatures seem to favor the expression of certain genes.

Transformations initiated by treatments with specific antisera are quite rapid. Much slower transformations can, in some cases, be effected by maintaining the Paramecia at higher or lower temperatures. In this way another gene may be favored and slowly have its antigen replace that of the formerly dominant gene. Our understanding of this beautifully analyzed situation is that before differentiation has occurred there are at least eight genes which might produce antigens. Once one, because the environment is more favorable for it than the others, has produced sufficient cytoplasmic antigen it is stabilized and dominant over the other members of the series.

This idea, that differentiation is stabilized by cytoplasmic substances, is an essential feature of several thoughtful suggestions concerning the nature of cellular differentiation (Rhoades, 1943; Sonneborn, 1943, 1950, 1951; Darlington, 1944, 1951; Haddow, 1944; Wright, 1945; Spiegelman, 1948; Brachet, 1949; Weiss, 1950; Ephrussi, 1951; Weisz, 1951).

The recent work of Billingham and Medawar (1948, 1950) indicates that cytoplasmic pigment granules of mammals may pass cell boundaries and perpetuate themselves.

Possibly the best evidence from multicellular organisms that complex cellular differentiation is stabilized in self-perpetuating cytoplasmic substances comes from a study of tumor agents. Already by 1914 the Rous group had three morphologically distinct tumors which could be transmitted by cell-free filtrates (Rous and Murphy, 1914). Each contained a specific agent which when injected into muscles of closely related chickens would not only cause growth but would direct differentiation along the same specific abnormal pathway. One of the tumors was a chondro-osteosarcoma and the agent from it introduced into young cells of the connective tissue of muscle not only caused those cells to grow but directed them to differentiate into the same abnormal type of cartilage and bone. Murphy (1935) pointed out very clearly that the complex differentiation of the three tumors is just as much the result of the agent as is the growth. He stated further, "This would indicate that the agent is probably closely related chemically to those factors normally determining cell differentiation."

Since the time when Murphy wrote, more of the nature of these growth-promoting determinants of abnormal cell type has been learned. Very few have been studied, but those which have are, or are associated with, the submicroscopic particles of the cytoplasm known as microsomes or possibly

in some cases with the larger mitochondria. The best characterized tumor agent is the one from the Rous Sarcoma I. It is a ribose-nucleoprotein-lipid particle approximately 70—100 mμ in diameter (Claude, 1940; Claude and Rothen, 1940). In conformity with the Murphy hypothesis it is now known that there are in all cells, normal and abnormal, microsomes of roughly the same dimensions. The Rous Sarcoma I and the Fujinami tumor agent, another particulate agent (Ledingham and Gye, 1935) are so similar to normal microsomes that they can be inactivated in vitro by antisera against normal chicken tissues (Amies, 1937; Amies, Carr and Ledingham, 1939).

Again in Murphy's words: "Formulated in a definite statement, the acceptance of the suggested interpretation means the assumption that the product of a characteristic cell (malignant) acts on an undifferentiated cell, causing it to assume the peculiar characteristics of the specialized one, and in this new form the cell produces more of the agent capable of inducing the transformation of other cells. Furthermore, the new cell, under the conditions of the hypothesis, must pass all of the properties to its descendants" (Murphy, 1935).

Recent work with amphibian agent tumors gives support to the hypothesis that tumor agents are modified normal differentiation agents. The common renal carcinoma of *Rana pipiens* of northern Vermont and adjacent Quebec contains an agent which is very specific (Lucké, 1938; Lucké and Schlumberger, 1949; Rose and Rose, 1952). It is effective only in closely related frogs and will grow only in kidney epithelium. However, it can become a temporary resident of young or regenerating cells of salamanders where its presence is indicated by unusual growth of the particular tissue in which it lodges. In three different experiments when it lodged in the periosteum of salamanders and caused hypertrophy of cartilage, an agent was recovered which in frogs induced not only renal tumors but also periosteal tumors. The new agents had retained their ability to live in and stimulate growth of renal epithelial cells, and, in addition, had acquired an affinity for the foreign tissue in which they had been temporary residents (Rose and Rose, 1952; Rose , 1952).

The specificity added to the original agent by sojourn in foreign cells is strikingly narrow. It is quite usual for a transformed agent to have received from its foreign host cell an affinity not just for cells of the same tissue but for cells of the same region of that tissue. An agent transformed by growth in a foreign cell is very likely to induce a secondary tumor in the exact spot on the other side of the body, giving rise to strikingly similar bilateral tumors.

These results indicate that there are regionally differentiated normal tissue agents which are sufficiently like tumor agents to make self-perpetuating combinations with them. Since it is the rule that nucleoprotein systems and even nucleic acids [chromosomes, various viruses, including bacterial viruses (Dëlbruck and Bailey, 1946; Luria, 1950), tumor agents (Berry and Dedrick, 1936) and even transformation agents of pneumococci (Taylor, 1949; Ephrussi-Taylor, 1951)] can form new self-

perpetuating combinations with other *closely related* nucleoproteins or nucleic acids, it seems very likely that both abnormal and normal differentiation involve the development of extremely specific cytoplasmic particles. These, from the facts mentioned above, can be seen to act as stabilizers of a very specific cell type. Various workers approaching the problem of abnormal differentiation and growth, along different pathways, have separately come to the conclusion that a change in self-perpetuating specific substances is the fundamental change in carcinogenesis (reviewed by Potter, 1945, and by Woods and duBuy, 1945, 1946).

The story of stabilizing self-reproducing nuclear-cytoplasmic systems is only a part of the story of differentiation. The most intriguing questions remain: how do the stabilizing particles become different in the various cell types and how do the elaborate patterns of differentiated tissues come into being?

OŌPLASMIC SEGREGATION

One of the first steps in pattern formation in an egg is the differential segregation of cytoplasmic materials (reviewed by Costello, 1948). Oōplasmic segregation is especially striking in mosaic eggs. In these, as shown by Raven (1948) for the mollusk, Limnaea, and Lehmann (1948) for the annelid, Tubifex, a great part of the cytochrome oxidase (indophenol oxidase) is allocated to the 2d and 4d cells. These two cells separated off in early cleavage stages are destined to produce most of the adult organs. Most of the other cleavage cells have a very limited supply of cytochrome oxidase and a limited lifetime, contributing only to embryonic or larval structures. The cytochrome oxidase occurs, as in other organisms, as small particles (Lehmann, 1950). Ephrussi (1951) has shown how strain differences in yeast result from differences in the cytochrome oxidase granules and has suggested localization of such particles as the basis of cellular differentiation in multicellular organisms. The comprehensive Brachet (1949) theory of differentiation also concentrates upon these granules as undergoing the fundamental change during differentiation. There can be little doubt that simple initial differences between blastomeres resulting from the segregation of cytoplasmic materials can eventually yield the expression of higher order differentiation.

In the mosaic systems where there is rather precise segregation of materials during cleavage, cells differentiate quite independently and induction is limited if not completely absent (Novikoff, 1938). Although induction or effective action between cells is strictly limited in the mosaic eggs, the 4d cell and its derivatives have much greater potency than exhibited in normal development. Penners (1936) has shown for Tubifex that extirpation of 2d, presumptive for the adult ectodermal structures, although resulting in failure of development of embryonic ectodermal bands, does not lead to absence of adult ectodermal organs, which in normal development always originate from those bands. Complete worms may arise from embryos lacking 2d cells. The 4d cell, presumptive for

endodermal and mesodermal tissues, is in a large sense totipotent, and can regulate to produce the extensive series of ectodermal structures: neural, excretory and locomotor organs. Strict mosaicism is confined to early development during the period of rapid differentiation of embryonic and larval characters.

THE ORIGIN OF QUALITATIVE DIFFERENCES ALONG QUANTITATIVE GRADIENTS

Localization of oõplasmic materials is not so precise in eggs of echinoderms and vertebrates, nor do the blastomeres develop as independently. Extremely minor differences and especially graded quantitative differences spreading and acting across cell membranes finally result in qualitative differences. In the amphibian egg the first epigenetic sign of differentiation is the appearance of the dorsal lip of the blastopore. In normal development and after a variety of experimental manipulations, the blastopore always appears at the circular, surface locus of junction of yolky and non-yolky materials. That part of the sub-equatorial circle which becomes the leader in invagination and various general metabolic activities was, many hours before, the point of greatest mixing along the circular locus (Ancel and Vintemberger, 1948; Pasteels, 1948). This dorsal locus within the marginal zone becomes the center of organization during gastrulation and neurulation. It has unlimited potency and can induce neighboring cells to complete the pattern of an embryo. The cells near its center, both endodermal and mesodermal, are the first to become capable of self-differentiation as tested by isolation (Holtfreter, 1938). This region in vertebrate embryos generally, like centers of organization wherever studied, leads in certain general metabolic activities. In the vertebrate egg it does not form from the original center of highest activity, but slowly becomes a secondary center of high activity, on the yolk-non-yolk locus. This organizer region does certain things sooner or faster than other regions. As far as we know, it is not qualitatively different before gastrulation. It is the first to invaginate, the first to utilize glycogen and pass to a respiratory quotient of 1, the leader in RNA production, the leader in phosphatase activity (reviewed by Brachet, 1949, 1950), and, in spite of the disagreement on this point, it may also lead in general oxidation-reduction activity. Spratt (Woods Hole Lecture, 1951) makes the point that the organizer region in the chick is a locus of high reducing activity.

In spite of many published objections to details of the Child (1941) theory of organization, the essential idea, and even it is not uncontested, is continually gaining support from laboratories all over the world. Child's idea that qualitative differences arise along, and as a result of, simple quantitative graded differences pervades embryological thinking. The clearest picture of qualitative differences arising in a gradient system comes from the echinoderm studies of Runnström (1928) and Hörstadius (1939 and earlier).

QUEST FOR THE ORGANIZER

During the years when the gradient concepts were growing, there was parallel development of the organizer concept (Spemann, 1938). In the latter there was emphasis on the fact that organization spreads from a point or small region. There is fairly good agreement now that the center of organization is a secondary development along one or more gradients (Runnström, 1928; Huxley and de Beer, 1934; Dalcq and Pasteels, 1937; Child, 1941 and earlier).

One result of the development of the organizer concept was a quest for specific inductors. After many fruitless years spent by many investigators in the search for specific inductors, the revolutionary work of Yamada (1937, 1940) has demonstrated the unspecific nature of the stimulus to differentiation emanating from the dorsal center of organization in the amphibian gastrula and neurula. For example, the developing notochord which lies in the primary center of organization does not produce something which acts specifically as an inductor of muscle, the tissue which normally differentiates adjacent to it. Instead, it acts unspecifically, stimulating differentiation and tending to raise the level of differentiation of any tissue near it to a more dorsal level. At a fairly late stage in the differentiation process presumptive blood from the far ventral side of an embryo can be isolated in an ectodermal jacket with a piece of notochord. The notochord at this late stage cannot raise the level of the partially differentiated ventral piece to the muscle level. Instead, the ventral piece under the action of the notochord can only achieve a lateral level and differentiate as pronephric tubules. If, however, the test piece at the beginning of the experiment is already a lateral piece destined to become pronephric tubules then notochord can raise it to muscle level of differentiation. In a masterly series of experiments by Yamada the fact comes out time after time that the stimulus from a differentiating region is general. Although cells at the center of organization are most effective in stimulating differentiation, other dorsal tissues are also stimulatory. For example, differentiating neural tissue can shift the differentiation of ventral mesodermal tissue toward a more dorsal type (Yamada, 1940).

LOSS OF TOTIPOTENCY

One of the bases of the hypothesis to be presented is the well known generalization that differentiation involves loss of potency.

In the extreme mosaic eggs totipotency is lost at first cleavage. This seems to be accomplished by segregation of oöplasmic materials as noted above. These materials, at least some of which are respiratory enzyme particles, need not be specific preformed organ-forming substances. They might, instead, as discussed below, simply confer upon their bearers the ability to perform a general reaction at a faster rate.

In most developing systems the apparent loss of potency is not so abrupt as it is in the strict mosaic eggs. Instead we find a slow progressive loss

of potency. At the other extreme from the strict mosaic systems are those in which the early cell divisions do not result in loss of totipotency in any of the regions. This is particularly well demonstrated by recent experiments on vertebrate embryos.

Ordinarily just one embryo develops from a blastoderm of fish or bird. More can be made to form from avian blastoderms by simply making isolating cuts across the blastoderm in situ (reviewed by Wolff, 1948). As many as six duck embryos have been produced from one blastoderm. Some of the regions, had they not been isolated, would have formed only extra-embryonic structures and blood. The important thing is that the totipotent parts be isolated from each other. Totipotency is rapidly suppressed once an organization center has begun to exert itself. This fact has been demonstrated very nicely by Luther (1936) for the trout.

All quadrants of a trout blastoderm in early morula and blastula stages are totipotent. All can, when isolated, produce all of the embryonic tissues. But as the dorsal region pulls appreciably ahead of the rest of the blastoderm, first ventral and a little later lateral regions lose the ability to differentiate. This is a constant feature of all organizing systems. They always show that as organization spreads, those regions away from the center are prevented from forming the central tissues. It may not always be as extreme as in the fish blastoderm where ventral and lateral tissues lose all ability to differentiate. These inhibited cells in the trout later regain the ability to form tissues as they move closer, during gastrulation, to the differentiating regions (Oppenheimer, 1936). One very important feature of organization is the progressive limitation of developmental pathways of originally totipotent cells as distance from the center increases.

Fish blastoderms can also be made to produce more than one embryo. The method is to treat the young blastoderm with any of a number of deleterious agents for a short time, or to decrease the oxygen tension or to keep the temperature low for a long period (Stockard, 1921). It is believed that these treatments greatly decrease the differences between dorsal and ventral regions. The observation supporting this is that two or three centers of gastrulation may develop simultaneously after the treatments and that each will become a center of differentiation.

Even the amphibian egg has recently yielded to treatment and whole embryos have been obtained from ventral parts of gastrulae and earlier stages when isolated from the dorsal organization center (Dalcq and Huang, 1948; Dalcq and Dollander, 1948; Dollander, 1950).

As these few examples show, parts of a vertebrate embryo are originally capable of forming much more than they do as part of the whole. All cells of the amphibian blastula, except the heavily yolked endodermal cells are capable of forming notochord when transplanted into the dorsal sub-equatorial region. One might think there is something qualitatively different confined to this dorsal sub-equatorial zone. Such is not the case. The whole region can be cut away in the early gastrula stage and adjacent

cells, presumptive neural and muscle cells, fill in the wound and differentiate as notochord (Holtfreter, 1938). The major question is why they would not have differentiated as notochord if the presumptive notochordal cells had not been removed. This is certainly a matter of inhibition by cells which are closer to the center of organization where differentiation begins. One can see how an originally totipotent system would necessarily differentiate if one region achieved a specific chemical reaction before other regions and if a product of the specific reaction passed to adjacent cells. Then, in the mass-action relationship that specific product would inhibit the same reaction in cells developing more slowly. This would be the beginning of specific differentiation. But what is the evidence that differentiated tissues are self-limiting?

In the course of development all normal tissues reach an equilibrium in which growth of a tissue no more than compensates for its loss. In the case of the friction epithelia and blood, considerable growth is necessary to maintain the equilibrium. The more is lost, the more will grow. On the other hand, the adult mammalian liver grows very little as evidenced by the rarity of mitosis. Is this because there is a low inherent growth potential in differentiated liver tissue? Not at all. If three quarters of the liver of a mouse is removed the remaining one quarter will grow very rapidly and reconstitute the original liver mass within one or two weeks. There is something very specifically liver involved in this rapid growth. When part of the liver is cut away the rest of the animal does not grow, just the liver grows. It seems that liver may produce a specific liver metabolite which builds up to inhibiting concentrations. That a specific liver metabolite courses in the blood stream may be inferred from parabiosis experiments. If mice are parabiosed and the liver of one partially removed, the livers of both will grow (Bucher, Scott and Aub, 1950). This is understandable if specific liver metabolites build up to growth inhibiting concentrations. That the growth control is effected by the concentration of a circulating metabolite follows from the fact that the uncut liver grew.

The same phenomenon has been demonstrated for neoplastic tissue. If a primary tumor and its metastases have almost reached an equilibrium where growth is slight, removal of the primary tumor may be followed by rapid growth of the metastases (Oberling, 1944).

These are examples of the principle that like inhibits like. This is a principle which is so well known that we are almost unaware of it. In regeneration studies it has been shown many times that the real stimulus to regeneration is the removal of a part. This is true whether the part removed be an apical shoot of a plant, the peristome of a ciliate, or a pint of blood. In regeneration studies emphasis has been placed on the stimulating effect of the severing cut. Because a number of easily observed things happen adjacent to the cut, the fact that the cut also removes a part is obscured. For example, when limb regeneration is studied in salamanders, much emphasis is placed on the wound changes after amputation. These are important and necessary, but one tends to forget that no amount

of wounding will stimulate regeneration of a hand on a limb if a hand is already present. Child (1929) was well aware of this fact and called the inhibition of like by like physiological dominance. Study of natural growth inhibitors is just beginning, but there is no doubt that they exist and that they play a role in differentiation. In plants where the study of differentiation is more advanced, it is recognized that although hormones are necessary for growth everywhere, apical auxin can suppress the growth of like but less well developed lateral shoot buds while stimulating the growth of unlike regions, for example, roots (Thimann, 1937).

In animals, also, although the work has not progressed so far as in plants, there is evidence of specific natural growth inhibitors. From a study of regeneration in Tubularia we know that unstable regeneration-inhibiting metabolites may be collected in the culture water (Rose and Rose, 1941). Apparently any region already producing anterior structures can prevent the formation of anterior structures in regions not yet producing them. It has been shown that the dominance is mediated by the fluid in the circulatory cavity (Barth, 1938; Rose and Rose, 1941) and can be explained as the work of inhibitors (Barth, 1944). This explanation is essentially an amendment to the older competition theory (Barth, 1941; Spiegelman, 1945).

A stem of Tubularia, like a young blastoderm of a duck, consists of totipotent regions. In both systems, unless the parts are isolated, one region will form certain structures and prevent all other regions from forming those structures.

A most instructive experiment by Miller (1937) on the very plastic Tubularia demonstrated that the position of the center of organization, which is also the dominant center, can be determined at will. This was done by raising the oxygen tension locally. The dominant center in regenerating Tubularia can also be determined by a locally higher temperature (Miller, 1939). In this originally totipotent system the region of highest general metabolism, whether it be determined by temperature, high oxygen level or low waste level, becomes the center of organization (Rose and Rose, 1941).

The center of organization at the base of a hydranth behaves very much like an amphibian organizer. In fact, the organizer concept, but without the name, was stated and demonstrated by Ethel Browne for Hydra in 1909 some years before Spemann and his colleagues demonstrated it so thoroughly in the amphibian embryo.

SIZE REGULATION

One more general principle with which a general theory of differentiation must be compatible should be recalled. This last principle is that the amount of any differentiated tissue is determined by the size of the whole. In the plant, Bryophyllum, the size of the regenerate is a function of the size of the leaf and stem from which it is developing (Loeb, 1924). The same is true for regenerating hydroids (Barth, 1938). The same principle

was observed in the developing amphibian embryo. Waddington (1938) added large amounts of ectoderm to gastrulae and found that not only were ectodermal derivatives enlarged but so were all other structures, including the notochord, which develops from the organizer region. In this experiment an addition of a large amount of undifferentiated material enabled all parts to be larger during differentiation. More cells became notochord, for example. Some of these, without the experimental interference, would have been limited to an inferior role in differentiation. More were able to play the leading role in the larger community. As will be shown below, this principle of the whole determining the size of the parts is understandable if each differentiating region can increase in size until its own products reach an inhibiting concentration. The larger the whole, the larger the part, provided the inhibiting metabolites can spread from their cells of origin.

THEORY OF A SELF-LIMITING HIERARCHY

The facts and generalizations presented above serve as the basis of a general theory of differentiation and pattern formation. Using as the model of general differentiation the special case known for Paramecia (Sonneborn, loci cited) and the Delbrück suggestion (see Sonneborn and Beale, 1949), one may imagine the process as it might operate in the amphibian embryo with a set of five genes, A—E, one of which is active in each of the following five tissues, notochord, somite, pronephros, mesothelium and blood. Each gene is thought of as in competition for substrate, S, and producing specific products, SA to SE, which come to occupy positions on cytoplasmic particles. Further, in order to have a workable differentiating system, it is necessary that specific products, possibly SA to SE, or products of them, $\frac{SA}{X}$ to $\frac{SE}{X}$, leave their cells of origin and pass through the entire system. A series of reactions involving the five genes can be written:

$$1.\quad S + A \rightleftharpoons SA \rightleftharpoons \frac{SA}{X}$$

$$2.\quad S + B \rightleftharpoons SB \rightleftharpoons \frac{SB}{X}$$

$$3.\quad S + C \rightleftharpoons SC \rightleftharpoons \frac{SC}{X}$$

$$4.\quad S + D \rightleftharpoons SD \rightleftharpoons \frac{SD}{X}$$

$$5.\quad S + E \rightleftharpoons SE \rightleftharpoons \frac{SE}{X}$$

In the amphibian gastrula the locus of highest general metabolism is an elongate meridional dorsal area. In this region all five reactions would be possible. One, with an inherent rate advantage, would be most likely to succeed. This is our reaction 1, the notochordal reaction. As we know,

any cell can become notochord, but we also know that not all of them will become notochord if they remain in contact with differentiating notochord. Consider now, cells which are near to, but to either side of, the most rapidly developing area. When their reactions 1–5 begin, there is a difference. Already reaction 1 in the cells in the most rapidly metabolizing adjacent region has produced some diffusible SA or $\frac{SA}{X}$. In the second region the situation is different from what it was in the first.

Second Region

1. $S + A \rightleftharpoons SA \rightleftharpoons \frac{SA}{X} + \frac{SA}{X}$ from first region.
2. $S + B \rightleftharpoons SB \rightleftharpoons \frac{SB}{X}$
3. $S + C \rightleftharpoons SC \rightleftharpoons \frac{SC}{X}$
4. $S + D \rightleftharpoons SD \rightleftharpoons \frac{SD}{X}$
5. $S + E \rightleftharpoons SE \rightleftharpoons \frac{SE}{X}$

The addition of $\frac{SA}{X}$ from the first region would tend to drive reaction 1 to the left, favoring the inferior reactions in the competition for S. The one which would succeed in establishing itself would be reaction 2 as the scheme is drawn. Cells somewhat farther away from the center on both sides and differentiating later would receive not only $\frac{SA}{X}$ but also $\frac{SB}{X}$, tending to drive both of their first two reactions to the left. This would give the third reaction of the hierarchy, the pronephric reaction, a chance. So differentiation would proceed until the cells of the far ventral region would have only one developmental pathway open to them, reaction 5, the one leading to the special products of blood cells.

The presence of a particular product in a region cannot be considered sufficient to cause a specific differentiation. Some confusion has arisen in differentiation theory because it has been assumed that the presence of auxin diffusing into a region should induce the same type of differentiation as was occurring in the region in which the auxin had been produced (Le Fanu, 1936). As the scheme is drawn, type 2 differentiation occurs not because $\frac{SB}{X}$ is present in that region but because reaction 2 is proceeding to the right and in turn determining collateral dependent reactions (not drawn).

The size of any part, and this means both the number of cells originally differentiating and the amount of subsequent growth of those cells, would depend upon the size of the whole. The larger the whole, if the specific

substances spread, the more cells could produce that specific substance before it had reached an inhibitory concentration in their vicinity, at which time a steady state would be reached.

As can be seen from the scheme, the specific product of one area, for example, $\frac{SA}{X}$, can be utilized in other areas. This, by increasing the supply of S, would stimulate differentiation unspecifically. This scheme does not require an original gradient, just one high region. Transmission of $\frac{SA}{X}$, etc., from the center would later establish a gradient. Simple diffusion probably would not account for sufficiently rapid spread of the specific metabolites. There is motion of cells during division and migration which would help in transmission. More likely, since in some systems only cells in intimate contact influence each other during differentiation, there is active transmission from cell to cell in these systems as there is known to be in the case of plants even before the vascular bundles arise.

We have been using a very early stage in differentiation as an example and have derived a bilaterally symmetrical system starting with five reactions and an elongate locus of highest general developmental activity. This leaves us far from the finished product. We know that separate qualitatively different sub-fields arise. Possibly the process as described above occurs in many fields leading to ever and ever greater specificity.

It also seems likely that once specifically different cells have arisen, complexity is increased by surface reactions comparable to antigen-antibody reactions (Tyler, 1947; Weiss, 1947; Burnet and Fenner, 1949).

As local specific differences increased, enzymatic adaptation to locally produced substrates would also be expected to multiply differences (Spiegelman, 1948). In time as the result of these various processes there might well be more different kinds of cells than there are genes.

Using the scheme presented above as a basis, one can predict that should a gene operating in one of the differentiation series mutate and produce a new type of viable cell after normal cells had differentiated, the cell bearing the mutant gene would be a tumor cell. It would be a tumor cell because it would be living in a society where the other members were inhibited by their own kind. The new type would not be inhibited greatly until it had produced more of its kind. In time many tumors (benign) do reach a growth-death equilibrium. In general those which do not reach an equilibrium are composed of more abnormal cells (reviewed by Willis, 1948). Presumably these are so unlike their parent tissue that there is very little mutual inhibition of parent and mutant tissue. Some of the most malignant tumors are composed of very abnormal cells with an average short lifetime. These are dying almost as rapidly as they grow. They may cause great destruction of tissue without ever achieving great mass.

It can also be predicted from the scheme that any mutation which increased the instability of the specific product would favor the growth of

the mutant tissue. This may be the case in some of those tumors which attain greater size than does normal tissue in tissue cultures. However, changed metabolism toward more efficient anaerobiosis may be the complete explanation in those cases.

The original change leading to neoplasia does not need to be at the chromosomal gene level. The fact that specific tumors can be induced by introduction of self-perpetuating cytoplasmic particles into normal cells means that mutation at the plasmagene level could also lead to neoplasia. That mutation at the plasmagene level without chromosomal mutation may occur is known for Paramecia (Dippell, 1950).

The major condition necessary for cellular differentiation according to the view expressed in this paper is an unspecific rate difference between one region and its environs. With the advent of specific reactions, a certain inherently favored reaction would become established in that region. Subsequently other lower order specific reactions would be established in cells adjacent to the cells differentiating in the center of organization. All cells except those very close to the center of organization would be channeled into inferior reactions by the diffusion to them of products of superior reactions. Differentiation would spread from the center until all cells had differentiated. The shape of the pattern would be determined essentially by the shape of the region of highest metabolism and by the diffusion or transmission pattern.

In such a differentiating system as drawn above, the regions already at the stage of specific reactions would stimulate differentiation of adjacent regions by releasing specific metabolites. These would diffuse and enter their specific reactions from the right and thus provide substrates utilizable in inferior reactions.

If differentiation were to be maintained after initiation in a gradient system, the gradient system would have to be maintained or differences would tend to decay. We know that they do not and also that the original gradient system is not maintained. Possibly the relegation of specific substances to self-perpetuating cytoplasmic bodies is the mechanism of perpetuation of differences. A specific substance may gain in chance of survival by becoming a member of an organized particle. This could be by exclusion of other specific substances from a favored position where a step or steps in synthesis occur. We know that the differentiating particles, microsomes and mitochondria and plastids, contain not only specific substances, but that they also contain energy transfer enzymes and nucleic acids associated with protein synthesis (reviewed by Brachet, 1949, 1950).

TEST OF HYPOTHESIS

After the above hypothesis of differentiation based on the interplay of initial rate differences and a hierarchy of self-limiting reactions acting through the mass-action relationship was formulated, a simple initial test of the general idea suggested itself. If the general idea is correct, one

should be able to suppress the differentiation of particular tissues by culturing embryos in suitable concentrations of products of the same adult tissues. This has been possible. Bloodless tadpoles have been obtained by culturing embryos through the neurula stage in a culture medium containing large amounts of living blood cells. Similarly adult brain will suppress the differentiation of neural tissue in frog embryos and adult heart will suppress the differentiation of embryonic heart. Details of the experiments are being published elsewhere.

SUMMARY

An hypothesis of cellular differentiation based upon the differentiation of cytoplasmic particles similar to tumor agents and differentiating like the surface antigen system in Paramecia as demonstrated by Sonneborn is suggested.

Various facts are assembled and used as the basis for a suggested mechanism of pattern formation.

Differentiation as it proceeds from a center of organization, seems to occur not as the result of release of a series of specific inductors, but by the release of a general stimulus upon which is superimposed a series of specific inhibitions.

One universal characteristic of differentiating systems is a progressive loss of potency. The loss increases with time and with distance from a center of organization. The center is characterized by higher general metabolism than any other qualitatively similar locus, and is the first region to do certain things.

Once an organization center has become active, other less rapidly developing regions are inhibited and do not attain the high level of differentiation proceeding in the center.

It is suggested that differentiation consists of the successive inhibition of one gene-initiated reaction after another by the collection of specific metabolites which inhibit in the mass-action relationship.

A major assumption in the hypothesis is that there are specific metabolites which in sufficient concentration will inhibit differentiation of the cell type which produced them. An initial test seems to support this view. Bloodless, heartless and nerveless tadpoles have been produced by culturing embryos with either living adult blood cells, or pieces of heart or pieces of brain in the medium.

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STUDIES ON POPULATION CHANGES IN BACTERIA AND THEIR RELATION TO SOME GENERAL BIOLOGICAL PROBLEMS

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A few years ago the results of earlier studies on mechanisms of population changes, frequently referred to as dissociation, in the bacterium *Brucella abortus* were reviewed in this journal (Braun, 1947a) and certain parallels between those results and other biological problems were discussed. Since then, considerable progress has been made in the analysis and understanding of factors involved in the variational phenomena contributing to population changes in Brucella cultures and it appears appropriate to review such progress at this time, noting again some relationships to general biological problems.

We have been concerned with one particular aspect of bacterial variation that has been observed extensively among pathogenic microbes, namely population change involving variants differing in colony form, culture characteristics, cell morphology, antigenic reactions, and virulence (cf. Braun, 1947b). Frequently, changes in these characteristics are linked, and, in conformance with established customs the types are usually labelled according to their colonial appearance as smooth (S), rough (R), mucoid (M), etc. In Brucella virulence and immunogenicity are almost always associated with the smooth colonial type. When smooth cells are inoculated into a liquid medium (complex or synthetic broth), plating of aliquots of the culture on an agar medium after various times of incubation will reveal the progressive establishment of non-smooth types in the originally homogenous population (fig. 1). Prior publications (e.g., Braun, 1947a, 1947b) reviewed experimental evidence that such population changes are due to mutation and subsequent selection of mutants best adapted to the existing environmental conditions. It was demonstrated that inherent differences in growth rates and viability of the parent type and in a variety of environmental conditions are capable of modifying the rate of population changes. Of particular interest were those environmental agents, e.g., normal serum (Braun, 1950), which were able to inhibit selectively the establishment of non-smooth mutants. Although these results had provided many data on the role of population dynamics in bacterial variation and had uncovered a variety of selective environmental conditions which affected population changes, the actual nature of the factors which restricted continued multiplication of the parent cell type while permitting the multiplication of mutant cells had remained obscure. Recent findings have uncovered the nature of at least one major factor involved and thereby advanced considerably our understanding of a number of variational phenomena.

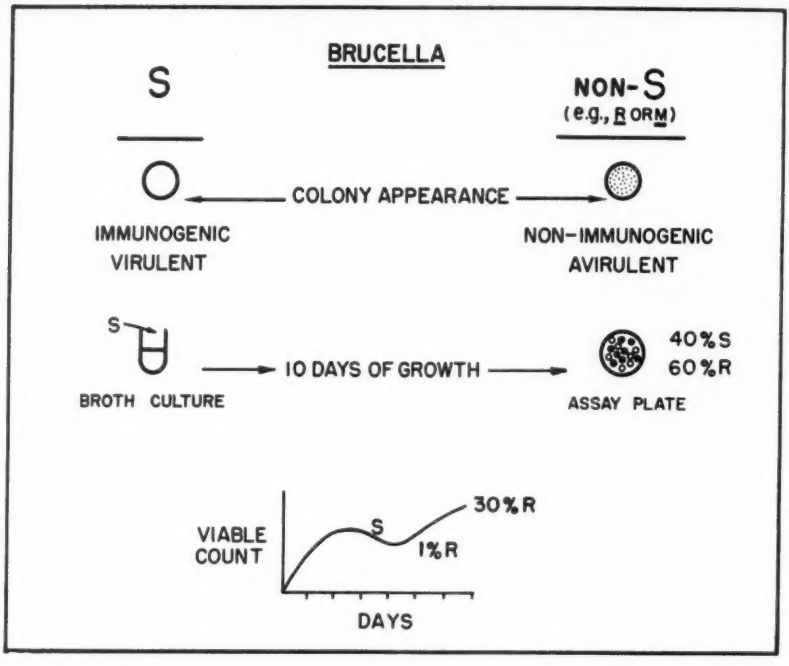

FIGURE 1. Diagram illustrating (a) properties of S and non-S types of *Brucella,* (b) population changes during prolonged growth of originally S populations, and (c) viable counts in originally S broth cultures.

EFFECTS OF METABOLITES ON POPULATION CHANGES

Brucella populations. The major advance was made when it was discovered that among the metabolites accumulating in the culture liquid of an originally S culture, there is a specific amino acid which inhibits the multiplication of the S cells but permits the propagation of more metabolite-resistant non-S mutants (Goodlow, Mika, and Braun, 1950). These observations were made with the help of a simple synthetic medium, containing DL-asparagine as the sole N-source, and paper partition chromatography of filtrates obtained from cultures of different age. It was found first that addition of filtrates from old cultures to fresh cultures inoculated with S cells only, would greatly enhance the establishment of non-S types, suggesting that the filtrates either contained a mutagenic factor or a component which favored the establishment of non-S mutants. Next, it was attempted to identify the responsible component in old cultures. Since asparagine and its breakdown product aspartic acids were the only amino-acids initially present, filtrates from cultures of various age were tested by chromatography for additional amino acids. It was found that an amino-acid with the Rf value for alanine appeared in the culture liquid just prior to the progressive establishment of non-S (usually R) mutants (fig. 2). Instead

of filtrate from old cultures, DL-alanine was then added to fresh S cultures and it was observed that DL-alanine enhanced population changes just as did filtrates from old cultures (table 1). Viable cell counts (plate counts) on "pure" S and "pure" R cultures in the presence of various levels of DL-alanine revealed that the increase of S type cells is inhibited by as little as 100 μg of alanine per ml, whereas R type cells remained unaffected until a concentration of 1,000 μg of alanine per ml of medium was reached. Subsequently, it was determined that these effects are due to D-alanine rather than L-alanine (Goodlow, Braun, and Mika, 1951). Once R type cells have established themselves in a population they will continue to produce alanine and thereby increase the alanine level in the culture sufficiently to yield concentrations inhibitory for R cells. Under such conditions the establishment of other, still more alanine-resistant, non-smooth mutants will occur, or a smooth type may establish itself which is identical in all respects with the original smooth type but differs from it in being highly alanine-resistant. This latter smooth type has been labelled S' and what had appeared to be a reversible population change, or the establishment of reverse mutants, from S to R and back to S, actually represented changes from S to R to S', the latter mutant possessing the high alanine

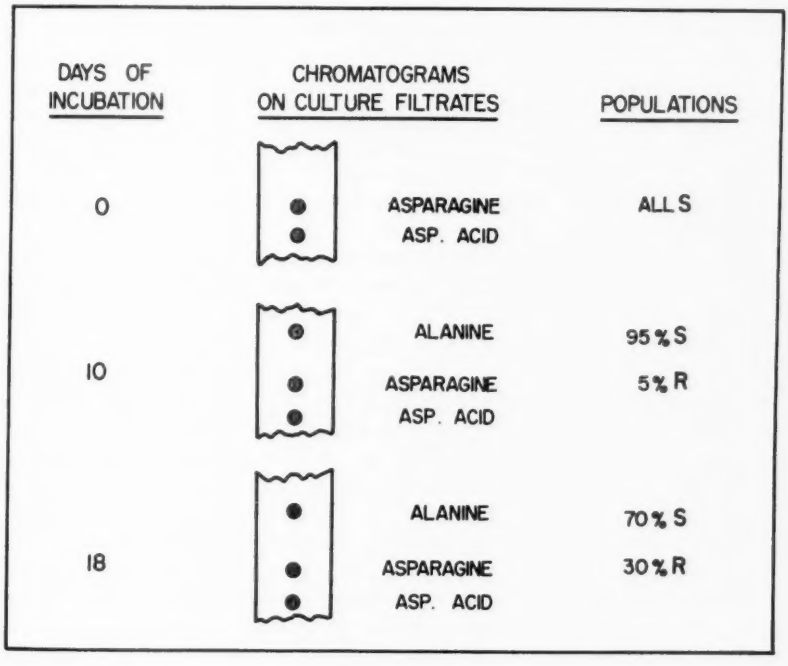

FIGURE 2. Accumulation of alanine and simultaneous population changes in originally S cultures of *B. abortus* growing in a liquid synthetic medium.

TABLE 1

POPULATION CHANGES IN ORIGINALLY S BRUCELLA ABORTUS, 6232, CULTURES AFTER ADDITION OF FILTRATE FROM OLD CULTURES OR ADDITION OF DL-ALANINE (SUMMARIZED FROM GOODLOW, MIKA, AND BRAUN, 1950)

Day of growth	Per cent non-smooth mutant cells			
	In plain synthetic medium	In synthetic medium plus old culture filtrate	In synthetic medium plus 100 μg DL-alanine/ml	In synthetic medium plus 200 μg DL-alanine/ml
0	0	0	0	0
2	0	0	0	0
4	0	0	1	2
6	0	1	6	19
8	0	2	14	36
11*	2	64	43	83
14*	6	91	57	86
19	76	91	....	....

*For columns 3 and 4 the days of observation were 10 and 15 respectively. Size of inoculum 6×10^6.

resistance required to give it a high selection value in old cultures (Goodlow, Braun, and Mika, 1951). Upon prolonged cultivation of cultures in which S' mutants had established themselves, additional population changes were observed which involved the establishment of a highly alanine-resistant dwarf S type, again phenotypically similar to S, and the subsequent establishment of a dwarf R type which appears to require alanine for growth.

Evidence has been obtained which indicates that *B. abortus* (Strain 19) produces alanine by transamination and direct amination (Altenbern and Housewright, 1951).

Additional evidence was obtained which indicated that alanine acts in a selective manner only and does not act as a mutagen. With the help of the fluctuation test (Luria and Delbrück, 1943) mutation rates to CO_2-independence were determined in the progeny of CO_2-requiring *Brucella abortus*, 6232, cells grown in cultures with or without added alanine (Mika, unpublished studies). Mutation rates were found to be identical, 4×10^{-10} per bacterium per division cycle, with or without prior exposure to alanine.

These data demonstrated that the accumulation of certain metabolites in the culture medium is responsible for the creation of the population pressure which has been recognized previously as a responsible factor in population changes. Prior to population changes in a closed system, such as the test tube, the parent type seems to create conditions which may be termed suicidal and thereby sets up specific environmental effects which will favor the selective establishment of metabolite-resistant antigenic mutants. In a way, then, these data suggested that the parent type actually influences the direction in which population changes may occur since its metabolic products determine which of the many possible spontaneously

arising mutants will have the greatest survival value at any given time of culture growth.

Experimental work has lent further support to this concept. It was noted that different S strains differ quantitatively and qualitatively in their metabolic activities and show corresponding quantitative and qualitative differences in population changes. It was observed also that, depending on the D- or L-configuration of the nitrogen source, metabolism and population changes may differ in cultures with similar inocula (Goodlow, Tucker, Braun, and Mika, 1951). Thus an S strain of *Br. abortus* showed rapid population changes and alanine production when grown in D-asparagine (plus vitamins and minerals of the synthetic medium described by Gerhardt and Wilson, 1948) but failed to show the establishment of non-S mutants or significant alanine production in an L-asparagine medium. Furthermore, an S strain of *Br. suis* grown in D-asparagine showed a rapid establishment of rough variants, whereas the same strain grown in L-asparagine exhibited population changes involving a mucoid variant, and simultaneously production of valine was detected during growth in the L-asparagine medium (table 2).

TABLE 2

POPULATION CHANGES AND METABOLISM IN ORIGINALLY S BRUCELLA SUIS CULTURES GROWN IN SYNTHETIC MEDIUM WITH D- OR L-ASPARAGINE AS THE SOLE NITROGEN SOURCE (FROM GOODLOW, TUCKER, AND MIKA 1952)

Form of asparagine	Percentage and type of mutant cells present on the following days					Amino acid metabolites in culture filtrate
	5th	8th	12th	16th	20th	
L	0	0	42 M*	52 M	41 M	Valine
D	16 R†	55 R	84 R	90 R	97 R	Alanine

*M = mucoid
†R = rough

Preliminary data have indicated that valine may act selectively in a manner previously described for alanine, inhibiting the multiplication of S cells and favoring the establishment of valine-resistant mucoid mutants in cultures containing L-asparagine as the sole nitrogen source. As might be expected, initially S *Br. suis* cultures grown in DL-asparagine media showed an accumulation of alanine and valine in the culture medium and establishment of both alanine-resistant rough and valine-resistant mucoid mutants. These results demonstrate that differences in the isomeric configuration of the nitrogen source in the culture medium can have a significant effect not only on the extent of population changes but also on the direction in which population changes may proceed.

Preliminary data also indicate that those environmental conditions which prevent the establishment of non-smooth Brucella mutants, for example,

addition of small amounts of normal serum or pyrophosphate, produce their effects by interfering with steps leading to alanine synthesis, thereby preventing the creation of an environment favoring the establishment of alanine-resistant non-smooth mutants. It is interesting to note that this selective effect of serum occurs only if sera from normal susceptible animals are employed. Sera from animals belonging to resistant species and from infected or vaccinated animals of susceptible species fail to produce similar effects (Braun, 1950); they must be assumed to lack the activity, found to be associated with components of certain globulin fractions (Cole and Braun, 1950a, Cole, 1950), that modifies the metabolism of S cells.

SELECTIVE EFFECTS IN OTHER BACTERIAL POPULATIONS

The role of metabolites in population changes may represent a general phenomenon among bacteria, even though aside from Brucella few cases have been analyzed along these lines. For example, there are reports on differences between virulent and avirulent types of *Mycobacterium tuberculosis* in their resistance to serine or alanine (Dubos, 1949; Marshak, 1951), differential resistance of virulent and avirulent types of crown-gall bacteria to the inhibitory effects of D-tryptophan (Hodgson, Peterson, and Riker, 1951), the development of alanine- and glycine-resistant strains of *Shigella dysenteriae,* the production of growth-inhibitory substances by *E. coli* and *A. aerogenes* (Coblentz and Levine, 1947), and the enhancement of population changes in cultures of *B. tularense* by as an yet unidentified factor in filtrates from old cultures (Eigelsbach, Braun, and Herring, 1951).

In addition, recent studies with *Salmonella typhimurium* (Page, Goodlow, and Braun, 1951) have yielded results similar to those obtained with Brucella. It was observed first that guinea pigs infected with a lethal dose of *S. typhimurium* showed a significantly shorter survival time if they were simultaneously injected with 50 mg of DL-threonine per animal. Inspection of cultures isolated from threonine-treated animals after death revealed the presence of a large proportion of cells giving rise to smooth-transparent (S^t) colonies in addition to cells which developed into smooth-opaque (S^o) colonies. In contrast, isolates from infected animals which had not been treated with threonine showed S^o cells only. Further studies (upper part of fig. 3) revealed that (1) S^t cells are more virulent than S^o cells, (2) S^t cells are more resistant to threonine than S^o cells, (3) a small number of S^t cells had been present in the original *Salmonella* culture, and (4) S^t mutants establish themselves also during *in vitro* growth of originally S^o populations, arising at a rate of approximately 1×10^{-6} per bacterium per division cycle (Page and Braun, unpublished data), and are favored due to the accumulation of threonine as a metabolic end product. The increase in virulence of *S. typhimurium* in threonine-treated animals, therefore, can be accounted for on the basis of originally heterogeneous populations containing small numbers of more virulent, threonine-resistant S^t types which will be selectively favored in threonine-treated animals. It is interesting to

note (lower part of fig. 3) that threonine injections failed to produce an effect on survival when animals were infected with homogeneous S° populations. Apparently, average time of death is too rapid (6 days) even after infection with the less virulent S° type to permit the selective establishment of S^t mutants arising at a relatively low rate. However, if a few S^t cells are present initially in S° populations, they will be selected immediately after infection of threonine-treated animals. These differences in results after use of initially homogeneous or heterogeneous populations of bacteria may account for the discrepancies in the literature (see Clark, 1950) regarding the effect of diet on infection.

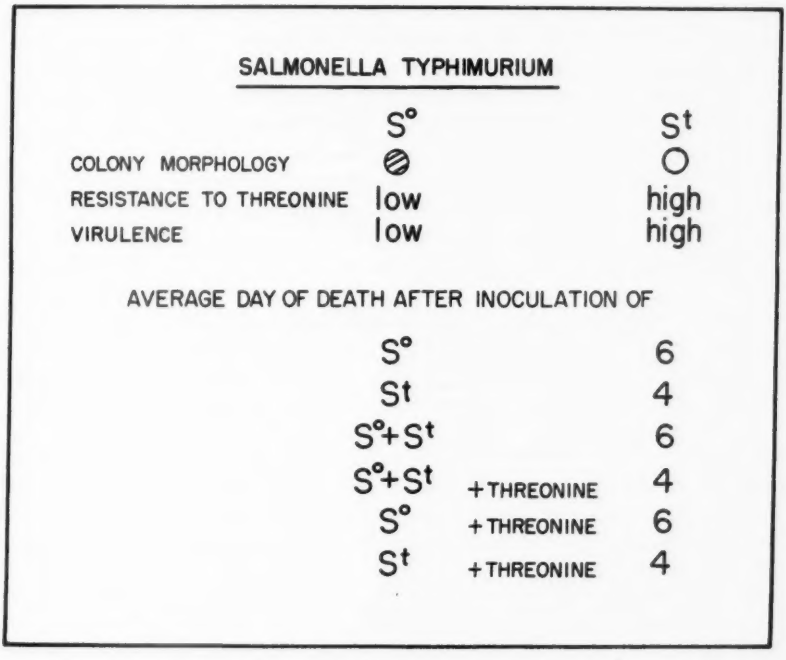

FIGURE 3. Diagram illustrating (a) properties of S° and S^t types of *Salmonella typhimurium*, and (b) the effects of injecting S°, S^t or mixtures thereof, with and without threonine, into guinea pigs.

POPULATION CHANGES OF BRUCELLA IN VIVO

Even though it was possible to influence population changes *in vivo* in the case of Salmonella by injection of threonine, under ordinary conditions the degree of population changes *in vivo* does not appear to be comparable to that *in vitro*. This became quite evident from extensive studies *in vivo* with Brucella (Braun, Gorelick, Kraft, and Mead, 1951). Using S clones which would produce extensive amounts of alanine and associated population changes during growth in test tube cultures, similar population changes could not be noted after infection of guinea pigs, even during stages when

the above mentioned selective serum effect had changed so that the establishment of non-smooth types was no longer inhibited *in vitro* after addition of serum samples from these animals. This difference between *in vitro* and *in vivo* results probably is due to the fact that *in vitro*, for example, in test tubes, we are dealing with what may be termed a "closed system," where the inhibitory metabolites can accumulate and favor the selective establishment of metabolite-resistant, less virulent mutants; in contrast, conditions *in vivo* may be compared to an "open system" from which metabolites are removed continuously. Experimental results favor this interpretation. Thus, it was found (Braun, Gorelick, Kraft, and Mead, 1951) that guinea pigs which had been infected first with highly virulent S cells and then were infected with less virulent but more metabolite-resistant M cells one or five weeks later, did not permit recovery of M cells from a great variety of tissues cultured two or more weeks after the M infection. However, localized abscesses were found in several of these animals and upon culturing revealed the presence of large numbers of M cells. Preliminary data revealed that, as one might expect *a priori*, abscesses contained higher concentrations of metabolites, including alanine, than were found in other tissues. Localized abscesses, therefore, may provide conditions similar to those present in a test tube, that is, a "closed system" which permits the accumulation of metabolites and thereby favors the retention of metabolite-resistant mutants, or their establishment if they arise by mutation in a more metabolite-susceptible S population. It is easy to visualize how differences between species or individuals in normally existent metabolite levels, in the host's efficiency of removing accumulating metabolites, or in the host's ability to affect metabolite production of the parasite may contribute to host susceptibility or -resistance by influencing the establishment of bacterial populations of different virulence *in vivo*. The medical literature contains many references to the isolation of variants with lowered virulence from chronic cases of various diseases and from resistant hosts (Braun, 1947b).

INTERACTIONS IN MIXED BRUCELLA POPULATIONS

Now let us return once more to studies with Brucella *in vitro*. The recognition of the role of metabolites in population changes made it possible to analyze and understand certain interactions in mixed populations. Previous investigators (e.g., Mohr, 1934) observed with several bacterial species that regardless of the initial proportion of S and R types in a mixed inoculum all cultures will display the same percentage of R types after a certain period of growth. Similar observations were made in early studies on the population dynamics of Brucella (Braun, 1947b) but the factors responsible for this attainment of an apparent population equilibrium[1] (table 3) had remained obscure. As soon as the role of alanine in population changes of Brucella was detected, this problem was reinvestigated (Braun, Goodlow, Kraft, Altenbern, and Mead, 1951). It was determined that S and R types

[1]Actually a "pseudo-equilibrium" which will be referred to as "equilibrium" in the discussion to follow.

differ not only in their susceptibility to inhibitory effects of alanine but also in rate of alanine production. S type cells produce alanine at a greater rate than R type cells. Thus a culture which initially contains a high percentage of S type cells will soon attain an alanine level favoring the establishment of R types. Consequently the percentage of R types will show a significant increase during a 10-day period of incubation. In contrast, there is much slower alanine accumulation in cultures which initially contain a high percentage of R type cells. In the absence of high alanine levels it is the S type which is selectively favored over the R type, consequently the percentage of R types will decrease in such cultures

TABLE 3

THE PERCENTAGE OF R TYPES IN 10-DAY OLD BROTH CULTURES OF BRUCELLA ABORTUS, STRAIN 19, INOCULATED WITH VARIOUS MIXTURES OF S AND R TYPE CELLS

	Each of the columns below represents the average result obtained on 3 independent cultures started with similar inocula					
Percentage R in inoculum	0	1	21	52	78	100
Percentage R in 10-day old cultures	18	17	20	21	19	100*

*Note that no S type cells established themselves during 10 days of growth in originally 100 per cent R cultures which may be attributed to the low rate of reverse mutation from R $\rightarrow$ S.

during the initial 10-day period of growth. As figure 4 illustrates, these population changes may take place very rapidly. Viable cell counts of pure S and R cultures grown in the absence or presence of DL-alanine (1000 μgm/ml) confirmed that S has a greater survival value than R in the absence of significant alanine levels, whereas R yields higher viable counts than S in the presence of alanine. These data demonstrate again that the selective value of any given variant may differ under different environmental conditions, but it is interesting to note that in the case analyzed here this shift in selective values can be referred to environmental changes produced by the cells themselves.

As had to be expected, the population equilibrium was found to differ when different S clones were used in experiments involving S + R mixtures. It can be safely assumed that such differences are due to differences in rate of alanine production of different S clones. This assumption is supported by the fact that those S clones which in initially mixed S + R cultures yielded equilibria containing higher percentages of R also exhibited a far more rapid establishment of R mutants in cultures inoculated with S cells only. The shift in S/R equilibrium that can be produced by inherent differences between S clones can also be brought about by environmental influences. Thus, initial addition of DL-alanine to cultures inoculated with S + R will result in S/R equilibria with larger proportion of R than

those found in control cultures without added alanine. Figure 4 illustrates such effects of different initial alanine concentrations in the cultures upon the selective advantages of the R type. The greater the initial concentration of alanine the greater is the proportion of R at 10 days. Preliminary data have indicated that if small amounts of alanine (less than 500 μgm/ml) are added to initially mixed S + R cultures, the population equilibrium is still detectable after 10 days of growth and is shifted in favor of a higher percentage of R types proportional to the initial amount of alanine present; if agents that interfere with alanine accumulation are added, the equilibrium will be shifted in favor of S type cells. If, however, alanine is added initially in amounts of 500 μgm/ml or more, mixed cultures with different initial proportions of R cells will fail to reach an equilibrium at 10 days.

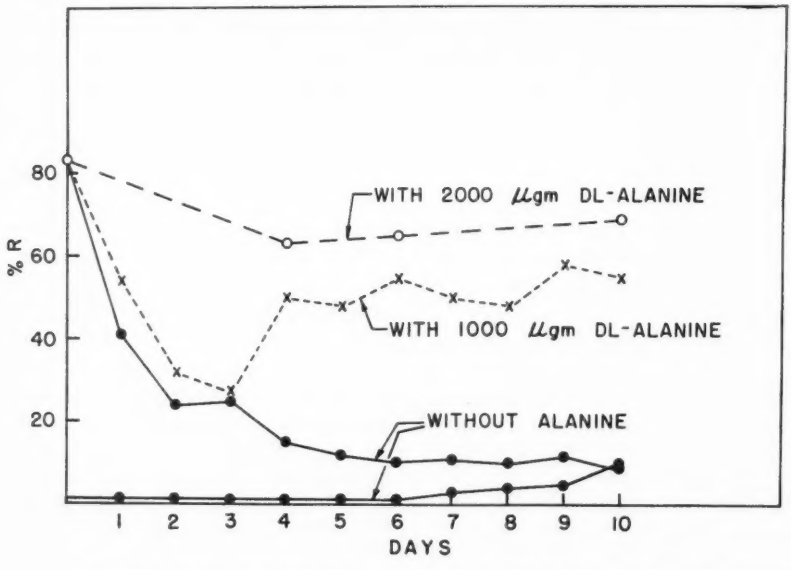

FIGURE 4. Daily changes in proportions of S and R types in broth cultures of *Brucella abortus*, Strain 19, started with mixed inocula, and the effect of initial addition of DL-alanine upon such population changes. Each point represents the average of three separate cultures.

DELAYED EFFECTS OF CHANGED ENVIRONMENT

One additional phenomenon detected in these studies is of interest, namely the existence of a "carry over" effect. By this is meant the ability of cells to continue, after transfer to new environmental conditions, reactions initiated under previous environmental conditions. For example, S cells previously grown in an alanine-free environment will grow for prolonged periods (approximately 8 generations) without being inhibited after transfer into media containing large amounts of alanine. This initial lack of an alanine effect is also noticeable in the curve showing popula-

tion changes in the presence of 1000 μgm DL-alanine in figure 4, where daily recordings were made which showed that even in the presence of added alanine the initial increase of S is similar to that in control cultures without alanine. This ability of S cells to withstand inhibitory concentrations of alanine for a limited period is probably attributable to the utilization of stored intermediates or co-factors (pantothenic acid?) for the performance of those vital metabolic steps with which alanine appears to interfere. Similarly, when S cells were pregrown for 3 days in media stimulating alanine synthesis, then mixed with R cells and transferred into plain media or media containing pyrophosphate which prevents S → R population changes presumably by interfering with alanine synthesis (Cole and Braun, 1950b), population changes proceeded initially just as under the prior environmental conditions that favored the establishment of R types. Also, pregrowth in pyrophosphate-containing media and subsequent transfer to plain media resulted in population changes which were initially identical to those found in pyrophosphate media. These delayed effects are reminiscent of the phenotypic delay observed during mutational changes (phenomic lag) part of which has been similarly attributed to a physiologic lag (Davis, 1949). A mutation resulting in the inhibition of a metabolic step may fail to manifest itself for some time due to the presence of stored intermediates that may yield the final product despite the lack of synthesis of early steps in the chain of metabolic events. This would be similar to the failure of alanine to inhibit Brucella S cells immediately after transfers from plain media. The apparent delay of alanine production that has been encountered after transfer of Brucella cells from pyrophosphate-containing media to plain media, akin to a phenomic lag of mutational steps involving the repair of an inactive metabolic process, is more difficult to explain, unless one assumes that initiation of synthetic processes may require time through the involvement of multiple threshold phenomena.

Before going on to a discussion of additional parallels between the Brucella studies and general biological phenomena it should be noted that interactions between metabolites and metabolite resistance, as in the case of Brucella populations, is apparently not the only mechanism which may lead to population equilibria. Atwood, Schneider, and Ryan (1951) obtained results in studies with auxotrophic and prototrophic *E. coli* mutants which indicate that in a constantly renewed environment periodic selection of mutants differing in selective values may lead to population equilibria. Similar data have been reported by Stocker (1949) and Novick and Szilard (1951). However, in the discussion to follow, only the phenomenon of population equilibria produced by interactions of metabolite levels and inherent metabolite-resistance, as illustrated in the Brucella studies, will be considered as a model for other biological problems.

COMPARISON WITH PARAMECIUM TRANSFORMATIONS

One such problem is the antigenic transformation of Paramecium (Sonneborn, 1948, 1949, 1950). Sonneborn and co-workers demonstrated that

antigenic variations of Paramecium are cytoplasmically controlled, but that the potential for manifestation of individual antigens is controlled by the presence of specific nuclear factors. Transformation from one antigenic type to another was accomplished by exposure to specific antisera, different levels of nutrients, or extreme temperatures. Once animals of one antigenic type had been transformed to another type they retained the new phenotype under standard cultural conditions through both sexual and asexual reproduction; yet the transformations are reversible under appropriate environmental conditions, demonstrating that no potencies are lost in the process of transformation. Despite this retention of multiple serotype potencies, only one of the antigenic factors is detectable in any hereditary type by serological methods. Sonneborn (1950) therefore has considered primarily two hypotheses for an interpretation of these phenomena: (1) variable gene activity (Kimball, 1947) where it is assumed that the presence of a particular product of gene activity stimulates the corresponding gene to greater activity, and (2) competitive processes among cytoplasmic reactions that may involve inhibition of some steps in the synthesis of all but one antigen-determining substance in the presence of high concentrations of one of the specific substances (hypothesis of mutually inhibitory alternative steady states as discussed by Delbrück in Sonneborn and Beale, 1949). More recently Beale (1952) has shown that at least in one variety of Paramecium, alleles at different loci are concerned with antigen production, and that the state of the cytoplasm, modifiable by environmental changes, selectively controls which gene can express itself. Neither Sonneborn's second hypothesis nor Beale's findings necessarily require a participation of self-duplicating cytoplasmic particles (Sonneborn, 1951; Beale, 1952) but do not exclude the involvement of such autocatalytic elements. If one assumes a role of competitive autocatalytic elements, either in the production of antigen-determining components of Paramecium or, as Beale (1952) has suggested, in the establishment of a particular cytoplasmic state, then certain parallels may be drawn between such competitive interactions and the above discussed population studies on Brucella. The Brucella data illustrated that on an intercellular level there exists a dependency of population equilibria upon metabolite levels. Similar phenomena might play a role on an intracellular level where populations of autocatalytic cytoplasmic elements may reach an equilibrium controlled by intracellular metabolite or amino acid levels. The element that represents the major proportion in such an equilibrium would then be responsible for phenotypic effects in the case of competition between antigen-determining components, or on Beale's hypothesis for the cytoplasmic state controlling selective manifestation of alleles at different loci. It is also quite possible that the cytoplasmic state merely reflects metabolic activity which in turn affects equilibria involving autocatalytic, serologically inactive, preantigenic elements and permits only the "majority" element, controlled by a specific gene locus, to develop into serologically active antigen. There are two additional factors that

make the comparison between the Brucella data and the Paramecium data attractive. First, the environmental influences known to cause antigenic transformation in Paramecium are all conditions that are known to affect metabolism, that is, temperature changes, nutrition, and presence of antisera (in studies with Brucella it has been observed that presence of antiserum significantly affects amino acid metabolism, an effect that is quite apart from antigen-antibody reactions). Second, the effect of these environmental agents upon antigenic transformations in Paramecium is a delayed effect, changes in antigenic characteristics frequently do not become detectable until several divisions have followed the period of treatment. This delay is closely paralleled by the delay and "carry-over" effects encountered when the action of metabolites upon population changes of Brucella was tested. As discussed above, these delayed effects may be associated with the presence of preformed intermediates (intermediates of antigen synthesis in the case of Paramecium ?) that can be utilized for some time even in the presence of inhibitory conditions which interfere with the formation of new products.

DIFFERENTIATION

The transition from these considerations to general problems of developmental differentiation is a simple step, especially in view of the stimulating discussions along these lines presented by Sonneborn (1950). Little can be added to what has already been stated regarding the implications of the role of the cytoplasm, as illustrated by the Paramecium results, in development of the spectrum of cellular possibilities inherent in embryonic cells. However, the results of bacterial population studies may suggest a mechanism by which various cytoplasmic controlling factors may exert their determinative influence. Similar to what has been stated above in connection with the determination of antigenic characteristics in Paramecium, metabolite levels may play a role in controlling the interaction and phenotype-determining concentrations of autocatalytic cytoplasmic elements. Such considerations would be in line with ideas advanced by Spiegelman (1948), specifically his concept of "differentiation as the evolution of a population of plasmagenes," and they may be equally applicable to various related concepts of differentiation, for example Waddington's conjectures on the role of competition in the snythesis of autocatalytic cytoplasmic protein (1948). The data, reviewed above, on interactions between cell types in bacterial populations not only furnish an adequate experimental model for the type of interaction that may occur between autocatalytic elements on an intracellular level but they also suggest that the forces responsible for such interactions may be represented by metabolite levels, particularly amino acid levels. Since the latter undoubtedly vary at different sites and from one developmental stage to another, depending to a large degree on the complexity of tissue differentiation existing at any given time, new conditions for cellular differentiation through shifts in the ratios of intracellular determinants

could be produced progressively. However, it is not necessary to restrict such considerations to events on intracellular levels only; intercellular interactions must play a decisive role as well. Once diverse cell types have arisen, their establishment during morphogenesis may be subject to control by existing metabolite levels. Such interactions between cell types and metabolites may be similar to the above cited observations on mixed bacterial populations where the ratio between two different types of cells was controlled by extracellular amino acid levels. As in an aging bacterial culture, metabolite accumulation and production probably differ qualitatively as well as quantitatively at different stages of ontogeny, depending on the types of cells present and their relative proportions. Such changing intercellular selective environmental conditions, therefore, could provide changing competitive conditions between cells of dissimilar differentiation. Competition as an element in morphogenesis has been considered by a number of investigators; a recent example is a paper by Dalcq (1949).

ORIGIN OF DIFFERENT CELL TYPES

The above considerations suggest a close parallelism between factors involved in the establishment of cells with dissimilar differentiation in morphogenesis and the establishment of genetically different cell types in growing bacterial populations. One difference between the two phenomena as discussed so far is the proposed mechanism for the origin of different cell types; namely undirected spontaneous mutation in the case of bacterial populations versus a more directed change due to cytoplasmic factors in the case of morphogenesis. The question now arises whether the parallels between bacterial population changes and developmental differentiation can be extended even further by assuming an undirected, spontaneous origin of tissue cells with unlike differentiation. This possibility is made attractive by the observation that *apparently* directed population changes can be caused in bacterial populations through control of the isomeric configuration of the N-source. As shown on p. 000, under the influence of specific amino acids produced by the parent cells, initially S populations can become either predominantly R or M depending on whether the S cells grow in D-asparagine causing an accumulation of alanine and subsequent selection of R mutants or whether they grow in L-asparagine which causes valine accumulation and selection of M mutants. One could visualize a similar selective establishment of certain cell types in morphogenesis under the influence of existing metabolites. However, in order to accept such selective differentiation based on random changes, it also becomes necessary to postulate frequent changes among developing cell populations in order to permit the selective metabolites to act on a sufficient assortment of variously differentiated cell types. Any selection from such a random assortment of cell types should produce, at least temporarily, a considerable residue of undifferentiated or dis-

similarly differentiated cells without selective advantage, a situation which does not seem to be reflected in experimental data. Therefore, if undirected genetic instability, subject to subsequent specific selection by metabolites, were a contributory cause in morphogenesis, some mechanism causing spontaneous cellular changes at rates far higher than encountered in most experimentally analyzed material would have to be postulated. These considerations and the available evidence from embryological studies indicate that the spontaneous, undirected origin of variously differentiated cells is less likely than some type of more directed change, yet recent evidence that many metabolites may also act as mutagens (D'Amato, 1950), and information such as obtained by McClintock on mutable loci in maize (1951), ultimately might remove some of the difficulties now envisioned in such a concept.

ORIGIN OF NEOPLASTIC CELLS

Finally, it has been pointed out (Braun, 1947a) that the parallels between bacterial population phenomena and other biological problems can be extended to the problem of neoplasms, where renewed growth usually occurs in fully differentiated tissues, that is, in the absence of such competitive factors as may normally exist during morphogenesis. It was stated in the previously published discussion that after initiation of renewed growth, the absence of such competitive forces in an adult organism may be responsible for the unchecked growth of neoplastic cells or their failure to adjust into regulated, harmonically differentiated tissue. The newer information which revealed that the accumulation of specific amino acids contributes to the competition between parent and mutant cells in bacterial populations, makes such speculation even more attractive because it is known that in higher organisms, metabolic disturbances, hormonal changes, and neoplastic growth coincide frequently. In fact, one recent assay on normal and neoplastic rat livers (Schweigert, et al., 1949) has revealed that considerable differences can exist in the amino acid content of normal and neoplastic tissue. However, it remains to be established whether such differences are merely a symptom or whether they reflect a causative mechanism of neoplastic growth similar to the selective action of amino acids in bacterial population changes.

SUMMARY

Recent studies on bacterial population changes, involving the selective establishment of mutants differing not only in colonial morphology, antigenic characteristics and virulence but also in resistance to specific extracellular metabolites (amino acids) produced by the parent cells, have been reviewed. Certain parallels between these phenomena and such problems as antigenic transformation in Paramecium, developmental differentiation, and neoplastic growth have been discussed.

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INHERITANCE OF PATHOGENICITY IN VENTURIA INAEQUALIS (CKE.) WINT.[1]

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The immediate purpose of this paper is to give a brief résumé of studies on the inheritance of pathogenicity in the apple scab fungus, *Venturia inaequalis* (Cke.) Wint. A longer range objective of the Wisconsin investigations that are here reviewed is to extend the study to some related fundamental problems of infectious disease. Special attention is, therefore, given to the materials and methods that have been developed and to their adaptations for researches on basic problems of infectious disease.

The nature of the disease reaction between the pathogen and its host is the central fundamental problem of infectious disease. The pathogen and the host are of coordinate importance in determining disease reaction. Each is variable under the influences of heredity and environment. Understanding and experimental control of these variables are of prime importance to the advancement of basic knowledge of the nature, development, and control of infectious diseases. While the details of variability may differ much from one disease to another and from animal to plant disease, the major principles concerned are similar throughout the entire range of infectious disease.

The most fruitful approach to the understanding of variability of organisms has been through genetic studies. For many years genetic studies relating to infectious diseases and their control dealt chiefly with the host. With advances in the knowledge of reproduction and inheritance in fungi, however, needs and opportunities for more attention to the pathogen have become increasingly apparent (Stakman, 1940a; Keitt and Langford, 1941). Fungus pathogens of plants offer exceptional advantages for such studies.

Substantial progress has already been made in genetic studies of phytopathogenic fungi (Craigie, 1940; Rodenhiser, 1940; Christensen, 1940; Stakman, 1940b; Johnson, 1946). Major attention was first given to the rust and the smut organisms (Craigie, 1940; Rodenhiser, 1940; Johnson, 1946). These pathogens have many excellent adaptations for such work. However, other fungi offer some important advantages that they lack. The investigations of Dodge and co-workers (Shear and Dodge, 1927; Dodge, 1927, 1928,

[1]The substance of a paper given in the Joint Symposium on Genetics of Microorganisms of the National Research Council and the American Society of Naturalists, sponsored by the American Phytopathological Society, the Mycological Society of America, and the Microbiological Section of the Botanical Society of America, Chicago, Illinois, December 29, 1947. Because of delays and withdrawals of manuscripts, the plan to print the papers of this symposium as a publication of the American Association for the Advancement of Science has been abandoned in favor of separate publication of the available manuscripts. This paper was brought up to date and submitted to The American Naturalist May 29, 1952.

and papers cited by Lindegren, 1942), Lindegren and colleagues (Lindegren, 1933, 1942), Beadle and associates (Beadle, 1945a, 1945b), and others (see Catcheside, 1951) have greatly advanced knowledge of certain nonpathogenic Ascomycetes in the genus Neurospora, and drawn attention to some rare adaptations of this type of Ascomycete for genetic and physiologic studies.

The work of the writer and associates and of others has shown that *V. inaequalis,* an eight-spored Ascomycete, has adaptations similar to those of the Neurosporas for genetic and physiologic studies and, therefore, affords unusually favorable material for researches on the inheritance of pathogenicity and on related problems.

Apple scab is a classical plant disease. As the most widespread and destructive malady of deciduous orchard fruit plants, it has been the subject of many investigations, and an extensive world literature concerning it has developed (Aderhold, 1896; Wallace, 1913; Keitt and Jones, 1926).

The work from this laboratory herein reported[2] was an outgrowth of studies begun by the writer in 1916 with the aim of improving methods of control for apple scab under the conditions encountered in Wisconsin. In order to advance this problem, it was necessary to make intensive studies of the epidemiology of the disease. This required isolating and studying the fungus *in vitro,* developing methods and special equipment for inciting the disease experimentally, and studying various major factors that influence its development. A key to progress was the incitation and study of the disease on potted apple trees in the greenhouse under controlled environmental conditions. Inoculation chambers were devised in which the relative humidity could be maintained at saturation at a wide range of controlled temperatures, and greenhouse units in which the inoculated plants could be incubated were held at various controlled temperatures. Results of these studies on the relations of environmental conditions to apple scab infection were reported by Keitt (1926) and Keitt and Jones (1926). Further development of equipment for such work was reported by Keitt, Blodgett, Wilson, and Magie (1937).

In studying the influence of environmental conditions on disease development (Keitt and Jones, 1926), it was noted that certain cultures of the fungus incited typical scab lesions on the leaves of some apple varieties, while on the leaves of others they incited mere flecks. Further studies on the variability of *V. inaequalis* were undertaken by Palmiter. Working independently, Wiesmann (1931) in Switzerland and Palmiter (1932, 1934) in Wisconsin found that different isolates of this fungus possess different pathogenic capabilities. Both of these investigations demonstrated that

[2]The papers of the writer and coauthors have been freely drawn upon. Grateful acknowledgments are made to the many coworkers who have participated in these investigations. These studies were supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation. More recently they have also been supported in part by a grant E-140 (C) from The National Microbiological Institute of The National Institutes of Health, Public Health Service.

isolates[3] of this organism may also differ considerably from one another in various morphological and physiological characters. This work was confirmed and extended by Rudloff (1934), Schmidt (1935, 1936a, 1936b, 1937), and Küthe (1935).

Nusbaum and Keitt (1938) made a cytological study of host-parasite relations of two monoconidial isolates of *V. inaequalis* and three apple varieties. The six isolate-variety combinations dealt with showed four distinct types of host reaction, very susceptible, intermediate, and two types of resistant. Spore germination, formation of appressoria or functionally equivalent structures, and direct penetration of the cuticle of the leaves occurred in all cases, without evidence of being influenced by the isolate-variety combination concerned. The details of penetration by the minute infection hypha and the subsequent development of the fungus in its characteristic position beneath the cuticle in close contact with the cellulose walls of the epidermal cells were described (*cf.* Wiltshire, 1915). In very susceptible leaves the host cells showed little abnormality until about ten days after inoculation, when progressive depletion of plastids and cytoplasm and increasing vacuolation began to appear in the upper palisade region at the middle of the lesion. This impoverishment slowly spread throughout the area underlying the fungus and was later followed by necrosis. The fungus showed no evidence of injury until the host cells had died. In leaves of intermediate susceptibility, impoverishment of the host cells was less rapid and severe and little necrosis occurred. In leaves of the resistant variety, growth of the fungus was sharply restricted; localized necrosis of epidermal cells, indicating hypersensitivity, occurred with one isolate but not with the other. The resistance of young leaves was attributed chiefly to relations between the fungus and materials emanating from the host cells, rather than to mechanical barriers. Illustrations of selected stages of penetration and lesion development were given for different isolate-variety combinations. It was shown that in suitable isolate-variety combinations the fungus, though confined to the subcuticular position, is able to derive nourishment efficiently from the underlying host tissues and to incite a wide range of pathological effects in them. It was pointed out that, in the ability to infect vigorous young host parts, in the diversity of isolate-variety reactions, and in the ability to live for many weeks in intimate relations with the living cells of a congenial host, *V. inaequalis* is strikingly similar to many of the so-called obligate parasites.

When it was shown that *V. inaequalis* comprises isolates, or bio-types, with strikingly different pathogenic capabilities, consideration was given to possibilities of employing this organism in genetic and related studies of some basic problems concerning the nature of parasitism and disease resistance. The work of Dodge and colleagues (cited above) and Lindegren and associates (cited above) had recently pointed out exceptionally

[3]The term "isolate" is used in this paper to denote an initial pure culture and its sub-cultures. In *V. inaequalis* single-spore isolates are haploid pure lines.

favorable adaptations of certain Neurospora species for genetic studies and had developed valuable methods.

The first problem was to determine whether the apple scab pathogen, which has many similarities to the eight-spored Neurosporas, has a functional sexual stage.

FIGURE 1. Section of a naturally developed perithecium of *V. inaequalis*. (× 760)

The development of the ascocarp of *V. inaequalis* (figure 1) had been studied by Killian (1917) and Frey (1924), whose findings were in general agreement though differing in some details. A brief summary of Killian's account follows:

The perithecium is initiated by a helical growth at the end of a hypha that is indistinguishable from vegetative hyphae. A coiled archicarp, or ascogonium, surrounded by an initial of a perithecial wall and provided with a well differentiated trichogyne, develops from this fundament. The apex of another hypha of the vegetative type develops into a well differentiated, multinucleate antheridium, which comes in contact with the trichogyne. A pore is formed in the walls, and the nuclei of the antheridium pass through the trichogyne into the ascogonial coil, which at this stage typically com-

prises about seven cells, each containing two to several nuclei. The septa of the ascogonial coil are dissolved and the antheridial nuclei mingle with those of the ascogonium. A tendency to pairing was observed in both the antheridial and the ascogonial nuclei before they mingled, and also in the mingled nuclei. Since the mingled antheridial and ascogonial nuclei were not differentiated from each other by the cytological methods employed, conclusions were not reached as to whether or not each pair comprised one nucleus from each of these organs. The middle cells of the archicarp give rise to multinucleate cells from which arises a system of ascogenous hyphae consisting of cells that are typically binucleate. Crozier formation occurs, the penultimate cell develops into the ascus, and its two nuclei fuse to form the primary nucleus of the ascus. The usual three nuclear divisions then follow, and eight ascospores are subsequently delimited. The mature ascospore, which consists of two uninucleate cells, may germinate and give rise to a vegetative mycelium composed of uninucleate cells.

Killian stated that it was seldom possible to trace the origin of the antheridial hypha, but that in one case it was definitely traced to the plectenchyma of the fusicladial stage. He concluded that, since the primary hyphae were originally widely separated, often growing from different surfaces of the leaf, the fungus is heterothallic according to Blakeslee's terminology. Kniep (1928), however, pointed out that this conclusion is unsubstantiated so long as it is not demonstrated that the respective sexual organs always arise from different mycelia that are derived from different ascospores.

Keitt and Palmiter (1938) approached the problem with genetic methods. The eight spores of an ascus of *V. inaequalis* were isolated and cultured *in vitro*. Since methods for producing the perfect stage of the fungus *in vitro* were not then known (*cf.* Herbst, Rudloff, and Schmidt, 1937; Jones, 1914), the isolates were cultured singly and in all possible pairings on the foliage of scab-free potted apple trees in the greenhouse, and selected leaves bearing the fungus were overwintered under natural conditions favorable for perithecial development. The methods and experience for successful conduct of this phase of the work had been gained in investigations reported by Keitt and Jones (1926), Keitt and Wilson (1926), Wilson (1928), and Keitt and Palmiter (1937). Perithecia that bore ascospores were found in all cases in which any of a group of four isolates was mated with any of the other four, but not when lines were used singly or paired within either group of four. The leaves of four apple varieties tested were typically infected by each of the eight isolates; those of two others were typically infected by four isolates, but not infected by the other four, save for the development of small flecks without sporulation of the fungus. When the isolates were paired *in vitro*, employing a medium containing malt extract and a decoction of dead apple leaves, and incubated at temperatures suggested by the results of Keitt and Jones (1926) and Wilson (1928), perithecia that bore ascospores were produced from four of the sixteen expectedly fertile pairings, but not in similar cultures of individual isolates or expectedly

non-fertile pairings. However, perithecial initials were formed in all cultures. Marginal conditions for producing the ascigerous stage *in vitro* were thus attained. It was concluded that *V. inaequalis* is heterothallic in the sense that the isolates studied fell into two groups with reference to sexual compatibility, being hermaphroditic, self-incompatible, intra-group incompatible, and inter-group compatible; and that combinations are, therefore, a major source of heritable variations in this pathogen.

In connection with the initiation of genetic studies, an examination of some nuclear phenomena of *V. inaequalis* was undertaken by Backus and Keitt (1940). Special attention was given to the ascus, vegetative mycelium, conidiophore, and conidium. The asci arose from croziers near the base of the ascocarp, the two nuclei of the penultimate cell fused to form the primary nucleus of the young ascus, and three successive nuclear divisions gave rise to eight nuclei as described by Killian (1917). The first-division spindles were oriented longitudinally in the elongated asci; those of the second and third divisions were longitudinal, oblique, or nearly transverse. The positions of the spindles were such that after each division the sister nuclei were ordinarily arranged in pairs in the serial order of occurrence of the nuclei from base to apex of the ascus. Though in some cases the nuclei or the ascospores that later developed were seen in a temporarily semi-biseriate arrangement, the spores always became uniseriate in the mature ascus. The cytological evidence indicated that sister ascospores ordinarily lie next to each other in the ripe ascus. This was confirmed by later work in which the ascospores were isolated in serial order and the resulting lines studied for various characters (Keitt and Langford, 1941; Keitt, Langford, and Shay, 1943). It was shown in this later work that, though minor changes in position of nuclei or ascospores are not infrequent, it is nearly always possible accurately to establish the pattern of nuclear descent. In the work of Keitt and Backus the ascospores seemed to be delimited as approximately spherical structures, at first uninucleate. After a nuclear division and a cell division, the ascospore at maturity was composed of two uninucleate cells of unequal size. The cells of the vegetative mycelium, the conidiophore, and the conidium were uninucleate (*cf.* Wiltshire, 1915; Killian, 1917; Frey, 1924). The results concerning development of the ascus and ascospores have much similarity to those reported by Wilcox (1928) for *Neurospora sitophila.* The fact that the vegetative cells of the mycelium, conidiophore, conidium, and ascospore of *V. inaequalis* are uninucleate is of much significance in relation to genetic studies with this organism, as this would seem to preclude the possibility of variability due to heterocaryosis such as commonly occurs in the Neurosporas and other fungi with multinucleate vegetative cells.

Keitt and Langford (1941) reported studies designed to lay a groundwork for further genetic investigations of *V. inaequalis.* This work was concerned largely with methods for isolating monoascosporic lines and culturing them *in vitro,* determining sex reaction, breeding the fungus *in vitro,* and testing the several lines for differential pathogenic capabili-

ties. Since the constancy of the characters dealt with and the reproducibility of results are of prime importance in genetic studies (*cf.* Brierley, 1922), special attention was given to these aspects of the work.

A method for isolating the ascospores with record of their serial order in the ascus was described. A perithecium was crushed in water and a free ascus of suitable maturity placed on a plate of clear, hard agar under a dissecting microscope. Two fine glass needles were used to remove the

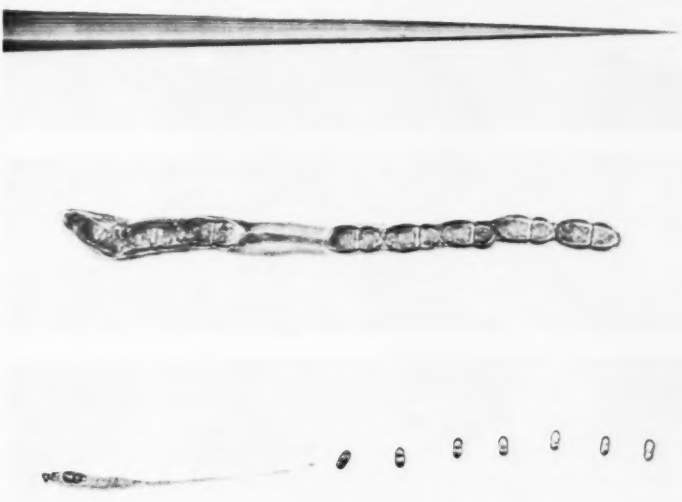
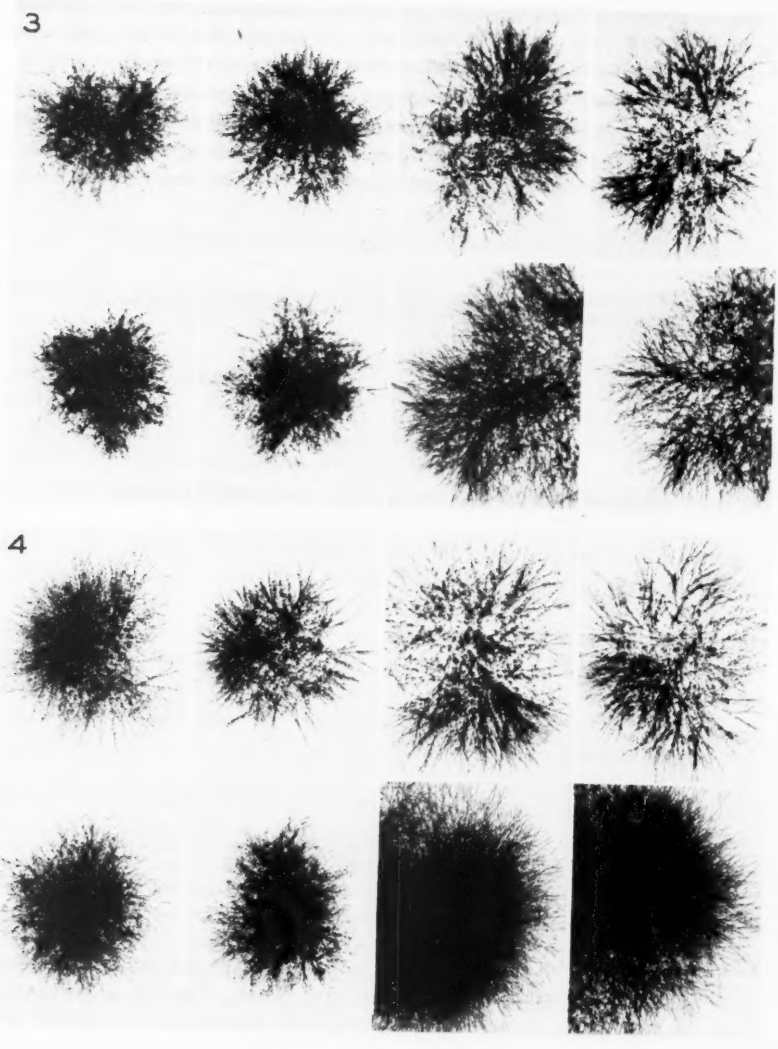


FIGURE 2. End of glass needle used in isolating the ascospores (× *ca.* 650); an ascus of the type from which isolations were made (× *ca.* 650); ascus with seven spores removed and arranged in serial order (× *ca.* 230). After Keitt and Langford, 1941.

spores from the ascus (figure 2). One needle, held in the left hand, pressed on the basal end of the ascus to keep it in place. The other, pressed behind the spores successively, beginning with the apical one, forced them out singly, and placed them in line in their serial order at intervals of about 1 mm. In some cases the eighth spore was left in the ascus. After all the spores had germinated, each was transferred to a plate containing 20 ml. of a malt extract agar. The isolates were numbered in the order of occurrence of the spores in the ascus, beginning with the apical one.

The eight spores from each of four asci were isolated with record of their serial order in the ascus, and the 32 isolates were studied for cultural characters under standardized conditions on malt extract agar. Successive monoconidial transfers of each line were made in triplicate at eight-



FIGURES 3–4. Photomicrographs of monoconidial colonies of the eight isolates from an ascus of *V. inaequalis*, after two weeks on malt extract agar at 16° C. The isolates in each figure are numbered in the order of occurrence in the ascus of the eight spores from which they were derived: upper row, left to right 1 (apical), 2, 3, 4; lower row, 5, 6, 7, 8. After Keitt and Langford, 1941.

FIGURE 3. Cultures of the third monoconidial transfer, photographed December, 1937. (× *ca.* 7)

FIGURE 4. Cultures of the thirteenth monoconidial transfer, photographed October, 1939. (× *ca.* 4)

week intervals, avoiding evident sectors if any were present. Photomicrographs after 14 days and photographs after eight weeks recorded colony characters. On the basis of colony characters, the eight isolates from a single ascus comprised four groups, each consisting of a pair of like isolates (figures 3-4). Each of the 16 pairs was distinguishable from every other pair by colony characters. The two members of any pair were indistinguishable from each other by any test tried.

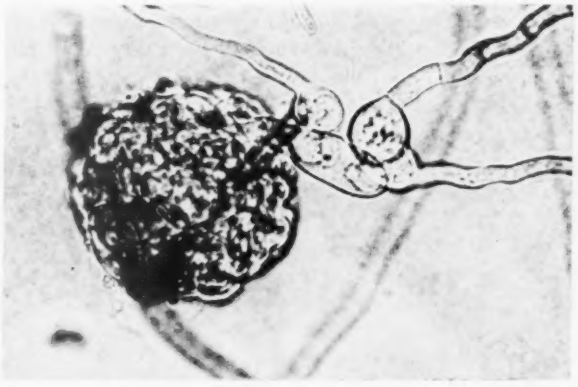


FIGURE 5. Perithecial initial from a malt extract agar plate culture of a monoascosporic isolate of *V. inaequalis*, showing the trichogyne and three antheridia. After Keitt and Langford, 1941. (× 730)

A method was developed whereby the fungus can readily be bred *in vitro* at suitable temperatures in plates of malt extract agar containing a decoction of dead apple leaves (*cf.* Keitt and Palmiter, 1938). The leaf decoction was shown to contain a thermostable organic component that stimulates the development of fertile perithecia. Sex reaction was determined by mating the isolate to be tested with each of two standard tester lines known to be compatible with each other. The 32 isolates studied were all hermaphroditic (figure 5) and self-incompatible. They comprised two groups of 16 (four from each ascus in each group), which were intra-group incompatible and inter-group compatible.

A standardized method was developed for testing pathogenicity of the isolates by conidial inoculation of leaves of potted apple trees, which were held in the inoculation chamber for two days at 16° C. and then incubated at temperatures held as near 16° C. as feasible in a greenhouse provided with equipment for cooling. The 32 isolates were tested on nine apple varieties. In each set from an ascus all eight isolates incited typical, sporulating lesions on one or more varieties, while on other varieties certain isolates of a set incited sporulating lesions and others did not. On differential varieties, it was usual for four isolates of a set to incite typical lesions and the other four to incite flecks (figure 6).

In the course of the work on the 32 monoascosporic isolates, about 1600 monoconidial cultures were grown on agar plates until they were eight weeks old. On these colonies 76 sectors appeared, as many as eight on certain lines, none on others. The frequency of sectoring was about the same after the cultures had been grown through 15 successive monoconidial transfers. Eleven lines were isolated (by single conidium or hyphal tip) from sectors and studied for cultural characters and pathogenicity in com-

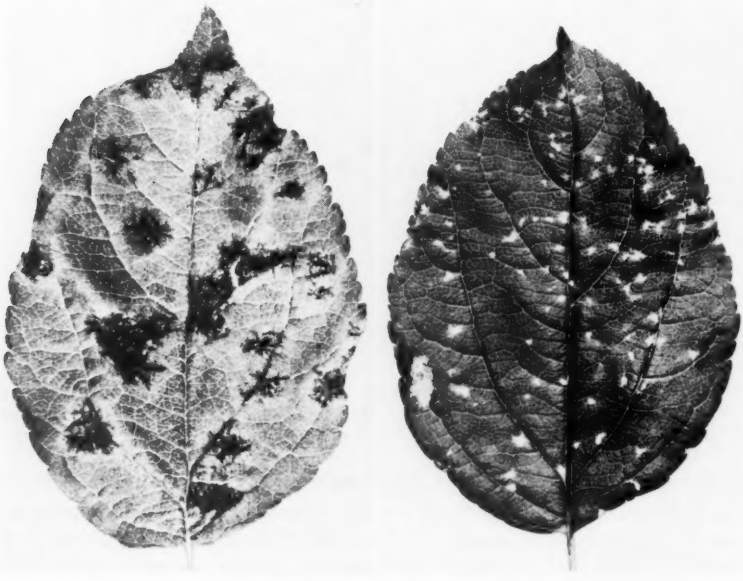


FIGURE 6. Apple leaves inoculated with *V. inaequalis*, showing characteristic lesion (left) and fleck (right) reactions.

parison with the lines from which they sprang. These sector lines varied widely from their original lines. All grew faster and produced fewer conidia; some bore no conidia. Many showed color differences, some being almost devoid of pigment. One incited white lesions. In no case did a sector line revert to the type of the line from which it sprang. The colony characters of sector lines showed no noteworthy change in one to three years in culture, except for the occasional occurrence of other sectors. All the sector lines were less pathogenic than the lines from which they arose, many of them infecting none of the nine varieties tested. Four successive host passages of several sector lines did not noticeably affect their colony characters or pathogenicity.

It was shown by these studies that isolates of *V. inaequalis* could be maintained *in vitro* for at least three years with a high degree of stability

of the characters under investigation, and that with the methods employed results were satisfactorily reproducible.

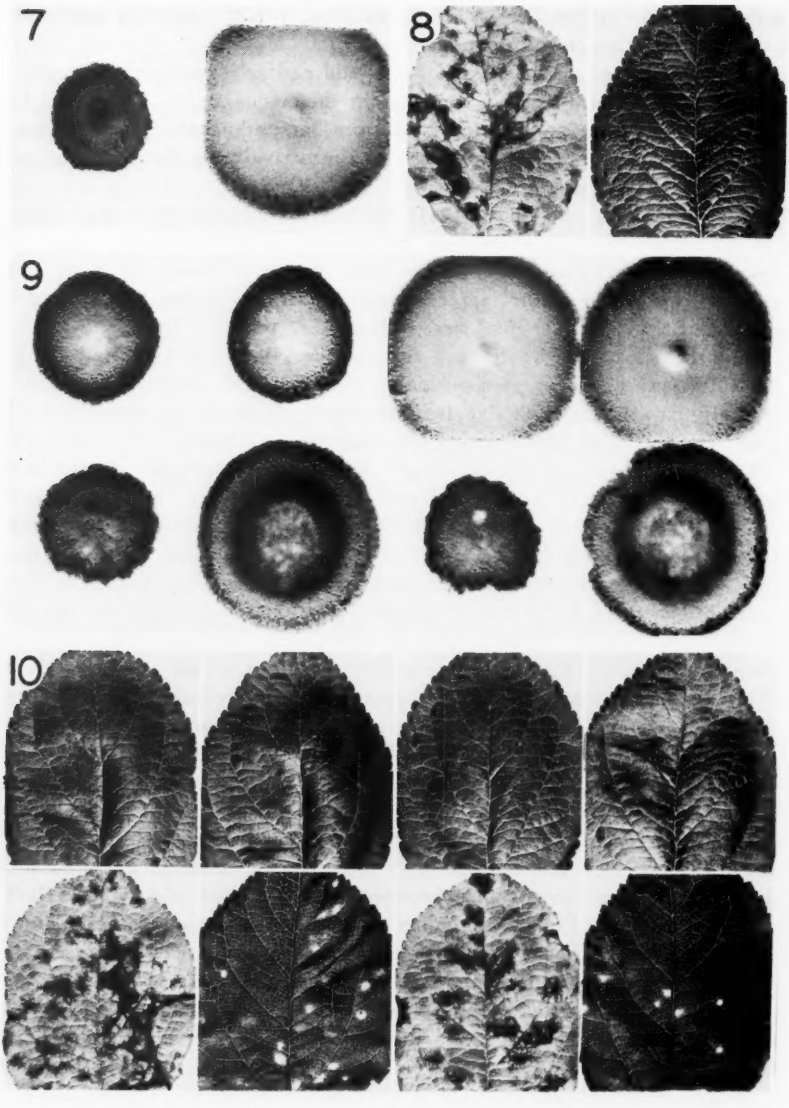
It was concluded on the basis of this and earlier investigations in this series that the third nuclear division in the ascus of *V. inaequalis* is equational and that segregation of factors for pathogenicity and sexual compatibility, respectively, may occur alternatively in the first or the second nuclear division.

Keitt, Langford, and Shay (1943) studied inheritance of pathogenicity and of certain mutant characters in *V. inaequalis,* employing selected monoascosporic isolates and mutant lines from the stocks of Keitt and Langford (1941) and several apple varieties. With minor modifications, the methods of Keitt and Langford were followed.

Only the two types of disease reaction described by Keitt and Langford (1941) were encountered with the wild-type lines studied, "lesion" (typically pathogenic, with abundant sporulation of the fungus) and "fleck" (non- or only slightly pathogenic, with sporulation lacking or very sparse). The lines were referred to as "lesion" or "fleck" according to the disease reaction incited. With wild-type lines and a given apple variety, crosses of lesion × lesion lines always gave all eight lines per ascus lesion; fleck × fleck, eight lines fleck; lesion × fleck, four lines lesion and four lines fleck. It was stated that in all cases in which adequate genetic analysis had been made in this series of investigations, segregation of factors for pathogenicity, as determined by the lesion and fleck reactions, had occurred in 1:1 ratio, alternatively in either the first or the second nuclear division in the ascus. The working hypothesis was advanced that the genes governing pathogenicity occur at one locus, with multiple alleles controlling different pathogenic capabilities to different apple varieties.

The probability was recognized that studies involving a greater range of isolates and apple varieties might reveal cases of more complex inheritance of pathogenicity in *V. inaequalis.* With different isolate-variety combinations, lesions and flecks of various sizes and characters occurred and disease reactions intermediate between typical lesion and typical fleck were easily found. For these studies on inheritance of pathogenicity, lines and varieties that gave well differentiated lesion and fleck reactions were chosen, first because they seemed to offer the simplest approach to the problem and also because a very close control of environmental conditions would be necessary for accurate differentiation of intermediate reactions from lesion or fleck.

Studies of progenies from crosses of the cultural mutant *tan* and the double mutant *tan nonconidial,* respectively, with *normal* (wild-type) showed that the genes controlling *tan* and *nonconidial,* respectively, segregated from their alleles in 1:1 ratio (figures 7, 9). In all cases studied, the presence of *tan* completely suppressed all macroscopic evidence of pathogenicity (figures 8, 10). *Nonconidial* also completely suppressed macroscopic evidence of pathogenicity, except in the case of McIntosh, with which the lesion reaction was modified to fleck (figure 10). Recovery of



FIGURES 7–10. Results from crossing the double mutant *tan nonconidial* with *normal* (wild-type) *V. inaequalis*. After Keitt, Langford, and Shay, 1943. (× *ca.* ½)

FIGURE 7. Parent isolates: left, *normal;* right, *tan nonconidial.* Eight-week-old cultures started, respectively, from a single conidium and a single hyphal tip.

FIGURE 8. McIntosh apple leaves inoculated, respectively, with the isolates in the corresponding positions in figure 7: left, sporulating lesions; right, no infection.

FIGURE 9. Progeny from crossing the isolates shown in figure 7: upper row, left to right, isolates 1 (apical), 2, 3, 4, lower row 5, 6, 7, 8, numbered in the order

the distinctive pathogenicity of the line from which *tan* and *nonconidial* arose, from the progeny of crosses of these mutants with *normal* lines of different pathogenicity, showed that the mutations concerned did not change the gene for pathogenicity.

The importance of guarding against errors in interpretation of the factorial basis of inheritance was emphasized, since it had been shown that either modifying genes or too high temperature could suppress the lesion reaction to a fleck or to no macroscopic expression. Adequate greenhouse facilities with suitable control of temperature and humidity are needed for work on this problem.

Schmidt (1940) reported experiments from which he concluded that inheritance of virulence in *V. inaequalis* to given apple varieties is complex. However, his tests of pathogenicity were conducted out of doors with limited opportunity to control environmental conditions, and he did not work with sets of eight lines stemming from individual asci. The differences in choice of isolate-variety combinations and in methods could well account for the differences between Schmidt's results and ours.

Shay and Keitt (1945) studied the inheritance of four mutant characters and sex reaction in *V. inaequalis,* with special reference to linkage relations and to the effects of certain of the mutant genes on the expression of pathogenicity. The mutant lines employed, *white, tan, nonconidial,* and *small,* were from the stocks of Keitt and Langford (1941).

By use of the method developed by Lindegren (1933, 1942) (*cf.* Zickler, 1934; Wülker, 1935; Mather, 1935; Ryan, 1943), based on the percentage of the asci showing second-division segregation of the gene-pair concerned, the distance in cross-over units from the locus to the centromere was computed for each of the four mutant genes and for the genes (alleles) governing sex reaction. Data on the percentages of parental and new combinations indicated that all five characters studied were inherited independently, except *white* and *tan.* The data from second-division segregation and new combinations were in close agreement in showing that the loci of the genes controlling these two characters are on the same chromosome arm, with *white* 6 and *tan* 10 cross-over units from the centromere.

It was shown that the effects of a single mutation on the behavior of the fungus may be varied and profound. The mutation to *white* prevented the development of dark pigment in the thallus, occasioned abortion of ascospores, and, probably by interaction with modifying factors, suppressed pathogenicity in the line from which it arose. Another mutant, *small,* suppressed macroscopic expression of pathogenicity, though penetration of the cuticle of the host occurred and there was a microscopic subcuticular de-

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of occurrence in the ascus of the spores from which they were derived; eight-week-old cultures, 1, 2, 5, 7 started from single conidia and 3, 4, 6, 8 from single hyphal tips; biotypes, *tan* (1, 2), *tan nonconidial* (3, 4), *normal* (5, 7), *nonconidial* (6, 8).

FIGURE 10. McIntosh leaves inoculated, respectively, with isolates in the corresponding positions in figure 9: isolates 1, 2, 3, 4 gave no infection; 5, 7 incited typical lesions; 6, 8 incited flecks.

velopment of the fungus. This work further emphasized the importance of adequate attention to the problem of modifying factors in the parental material before undertaking a factorial analysis of inheritance of pathogenicity of this organism.

Further studies on the inheritance of pathogenicity in the apple scab fungus were reported by Keitt, Leben, and Shay (1948). The primary objective was to test the multiple allele hypothesis proposed by Keitt, Langford, and Shay (1943); with minor modifications their methods were employed. A monoascosporic wild-type line of *V. inaequalis* that incites typical scab lesions on the leaves of the Haralson and Wealthy apple varieties and flecks on those of Yellow Transparent and McIntosh was crossed *in vitro* with each of two monoascosporic wild-type lines that incite the converse reactions, fleck with Haralson and Wealthy, and lesion with Yellow Transparent and McIntosh. The eight ascospores were isolated in serial order from each of 35 progeny asci, and the resulting lines were tested for pathogenicity to each of the four apple varieties. In each ascus tested, segregation for pathogenicity to each variety, as indicated by the lesion and fleck reactions, was in 1:1 ratio. The results of this work did not support the multiple allele hypothesis for the inheritance of different pathogenic capabilities in *V. inaequalis*. On the contrary, they showed that one pair of genes (or possibly more than one pair of very closely linked genes) governs inheritance of pathogenicity of these lines of the fungus to the leaves of Haralson and Wealthy and another pair governs inheritance of pathogenicity to Yellow Transparent and McIntosh. There was no evidence of linkage between these gene pairs.

Keitt and Boone (1951), working with a cross of two selected wild-type lines of the fungus and with four apple varieties, showed that three lesion/fleck gene pairs, respectively, conditioned pathogenicity to certain varieties, one pair to Haralson, another to McIntosh and Yellow Transparent, and a third to Red Astrachan and Yellow Transparent. The fleck allele of each of the two gene pairs conditioning pathogenicity to Yellow Transparent was epistatic to the lesion allele of the other.

Keitt (1952b) reported that further work had revealed another gene pair conditioning pathogenicity to Haralson. He stated that incomplete data strongly suggested two more loci for conditioning pathogenicity and pointed out the probability that many more can be found. Unpublished results of Keitt and Boone have confirmed these two loci, bringing to six the total number of gene pairs thus far shown, respectively, to condition pathogenicity to the leaves of a certain apple variety or group of varieties.

As pointed out by Keitt, Leben, and Shay (1948), each gene pair shown to govern pathogenicity to the leaves of an apple variety becomes a potential tool for further studies of the inheritance and the nature of pathogenicity.

The single-gene control of pathogenicity so often demonstrated in these studies raises the question of the physico-chemical basis of this control. Though this question cannot be satisfactorily answered in the

present state of knowledge, the numerous cases of single-gene control of specific biochemical reactions that have been demonstrated with Neurospora (Beadle, 1945a) and other organisms (Beadle, 1945b) suggested to Keitt, Leben, and Shay (1948) the working hypothesis that critical single-gene-controlled biochemical reactions condition pathogenicity of *V. inaequalis*.

Though the present paper is concerned chiefly with the inheritance of pathogenicity, a major purpose of the Wisconsin investigations here reviewed has been to develop materials, methods, and points of view that might open the way for investigation of other basic problems of infectious disease, such as the nature of the disease reaction between pathogen and suscept. The work accomplished has shown that the apple scab disease affords unusually favorable materials for such investigations. Consequently, our program has been broadened to include correlated studies on the genetics, nutrition, and pathogenicity of *V. inaequalis*. Early progress of these studies has been reported in part (Leben and Keitt, 1948; Boone and Keitt, 1949; Boone, 1951; Keitt, 1952a) and briefly reviewed elsewhere (Keitt, 1952b). These investigations are being continued.

SUMMARY

It was shown in the work here reviewed that *Venturia inaequalis* (Cke.) Wint., the Ascomycete that incites apple scab, has advantages similar to those of the eight-spored Neurosporas for related genetic and physiologic studies, and therefore affords exceptionally favorable material for investigation of basic problems of infectious disease such as the inheritance of pathogenicity, the origin and stability or plasticity of biotypes or races with different pathogenic capabilities, and the nature of the disease reaction between pathogen and suscept. Some specific advantages shown for this fungus for such studies are: (A) It freely infects young, vigorous, unwounded tissues and may live for many weeks in intimate association with living host cells. (B) It has many biotypes with different pathogenic capabilities to different host species or varieties. (C) It can be cultured throughout its life cycle and cross-bred at will *in vitro*. (D) It can be maintained for many years *in vitro* with satisfactory stability of the characters studied. (E) It is haploid throughout the parasitic phase and in its vegetative stage *in vitro*, thus permitting study of effects of a single set of genes. (F) The ascus contains the complete progeny stemming from a single meiosis, with survival of all nuclei in an orderly arrangement that permits tracing the line of nuclear descent by isolating the ascospores in their serial order and studying the derived cultures. (G) The vegetative cells are uninucleate and free from important problems of heterocaryosis. (H) The fungus responds well to mutagenic agents. (I) Materials and methods have been developed for correlated studies on its genetics, nutrition, and pathogenicity. A limitation on the use of this material in many places is the necessity for adequate greenhouse facilities with suitable control of temperature and humidity.

Only two main types of pathogenic reaction were encountered with monosporic wild-type lines of the pathogen, "lesion" and "fleck." The lesion reaction was typically pathogenic with abundant sporulation of the fungus, the fleck non- or slightly pathogenic with sporulation lacking or sparse. With a given apple variety the fungus lines were referred to as lesion or fleck according to the reaction incited. In lesion × fleck crosses pathogenicity to a given variety ordinarily was conditioned by a single lesion/fleck gene pair, which segregated alternatively in the first or the second nuclear division in the ascus. Each lesion/fleck gene pair studied conditioned pathogenicity to one group of apple varieties and not to another group. Six such gene pairs were found and occurrence of many more was hypothesized. Two of these gene pairs were shown separately to control pathogenicity to one apple variety; the fleck gene of each pair was epistatic to the lesion gene of the other. The working hypothesis was advanced that critical single-gene-controlled biochemical reactions condition pathogenicity of *V. inaequalis*. The probability was recognized that studies involving a greater range of isolates and varieties would reveal cases of more complex inheritance of pathogenicity.

The importance of guarding against errors in interpretation of the factorial basis for inheritance of pathogenicity on account of modifying factors in the parental lines or of inadequate control of experimental materials or conditions was shown.

It was concluded that the species *V. inaequalis* comprises a great number of biotypes with differential pathogenicity to various apple varieties and *Malus* species and that a major source of such biotypes in nature is genetic combination.

Cultures of the fungus were carried *in vitro* for some 15 years without evidence of loss or gain in pathogenicity.

The Wisconsin research program has been broadened to include correlated studies on the genetics, nutrition, and pathogenicity of *V. inaequalis*, some early results of which have been reviewed elsewhere (Keitt, 1952b).

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REDUCTION IN PRODUCTIVITY AND RECESSIVE LETHAL MUTATION FOLLOWING X-IRRADIATION OF FEMALE *Drosophila melanogaster**

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It is surprising that relatively little is known concerning the induction of mutation in the female germ line of *Drosophila melanogaster,* when one considers the large body of available information concerning mutation in sperm following irradiation. Although the sex-linked lethal mutation rate of female *Drosophila melanogaster* had been studied previously, it was noticed first by Gall in 1950 that the initial eggs laid by X-irradiated females contained higher frequencies of sex-linked recessive lethal mutations than those laid one week later. He suggested that the decrease in the recoverable mutations resulted from the selective elimination of lethals associated with chromosome rearrangements. The experiments to be described confirm and extend the observations of Gall.

MATERIAL AND METHODS

In our experiments Canton-Special female wild type *Drosophila melanogaster* freshly hatched or 2-3 days old were irradiated in gelatin capsules with 4,000 r of 100 kv X-rays (30 ma, 0.25 mm Cu, 1 mm Al, h.v.l. 0.5 mm Cu, 14 cm T.S.D., 269 r/min, 14.8 min. exposure). Sex-linked recessive lethal mutations were detected by the Muller-5 method. Irradiated virgin females belonging to either age group were mated in separate ¾ oz. creamers to M-5 males. The mating pairs were transferred to fresh creamers on six successive occasions; each transfer being separated by a 24-hour interval. After the seventh transfer the mating pairs were left for five days in fresh creamers and were then removed and discarded. Consequently F_1 individuals from the seven series of cultures represented eggs laid by treated females 0-1, 1-2, 2-3, 3-4, 4-5, 5-6, 6-7, and 7-12 days after irradiation. The successive batches of eggs in turn represent cells which were at increasingly early stages of oogenesis at the time of exposure to X-rays. Similar transfers of untreated mating pairs were made. The resulting cultures served as controls in the study of the reduction of productivity produced by the treatment.

F_1 females were allowed to mate in mass with their brothers and were then transferred to separate creamers. The progeny of each F_1 female was examined through the side of the creamer at a magnification of 7X with a dissecting microscope. The absence of red-eyed males in cultures containing a minimum of 15-20 M5 males was taken as the criterion for the pres-

*Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

ence of a sex-linked recessive lethal. A record was kept of the average number of F_1 flies per culture from X-rayed and control P_1 females of the freshly hatched and 2–3 day old categories.

RESULTS

A total of 188 sex-linked recessive lethal mutations were recovered from 2,822 tested egg X-chromosomes. The frequency of lethals, 6.7 per cent. shows a highly significant difference from the value of 9.9 per cent. derived from the data of Spencer and Stern (1948), who recovered 182 sex-linked recessive lethals from 1843 tested sperm X-chromosomes irradiated with 4,000 r of 90 kv X-rays. As in this work the Muller-5 method was used and the Canton-Special stock treated.

TABLE 1

Time interval (days) between irradiation and egg production	X-Chromosomes tested	Number of lethals	Per cent of lethals
0–1	25	2	8.0
1–2	151	16	10.6
2–3	158	12	7.6
3–4	149	14	9.4
4–5	269	20	7.4
5–6	240	18	7.5
6–7	314	20	6.4
7–12	1516	86	5.7

Table 1 shows the distribution of the 188 lethals among the samples of eggs tested. This table includes data from P_1 females of both age categories. The data for the first two time classes were lumped together and the frequencies of lethals plotted against time using the mid point of each time class (fig. 1). The straight line given by the equation $y = 0.0986 - 0.0045x$ is found to fit the points satisfactorily. The total sum of squares of deviations from the mean of the frequencies of lethals found is 0.5328. This can be converted into a Chi-square for 6 degrees of freedom by multiplying 0.5328 by $(2822)^2/188$ (2822–188). This total sum of squares of deviations can be divided into two portions; that due to regression of the frequencies of lethals in the eggs laid on successive days and that due to deviations from that regression. The sum of squares attributable to linear regression $= [\Sigma fy^2 - (\Sigma fy)^2/\Sigma f] - \Sigma f(y - y')^2$

where f = the number of X-chromosomes tested in each time class;
y = the frequency of lethals in each time class;
$y - y'$ = the deviation of the observed frequency from the frequency determined by the line of best fit for each time class.

A value for Chi-square of 7.50 is obtained after substituting the appropriate values into the equation: X^2 = (total chromosomes tested)^2 times the

sum of squares attributable to the linear regression divided by total lethals recovered times the number of lethal-free chromosomes. The P value for a Chi-square of 7.50 for one degree of freedom for linear regression is less than 0.01, thus indicating a highly significant reduction in recoverable lethals from eggs irradiated at progressively early developmental stages.

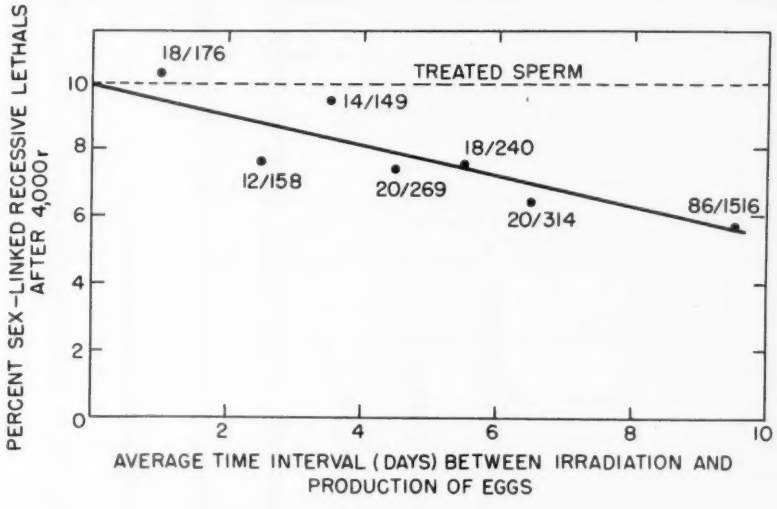

FIGURE 1

The data collected concerning the sterility induced in treated females are presented graphically in figure 2. The eggs laid in one week by females irradiated shortly after emerging from their puparia (Series A) produced F_1 populations 32 per cent. as large as the F_1 populations produced under identical conditions by untreated females. Female flies 2–3 days old when irradiated (Series B) produced F_1 populations 43 per cent. as large as the controls. The sex ratio in all cultures was about 49 per cent. males. In Series A 35 cultures from treated P_1 females and 39 cultures from control P_1 females were examined in each of the eight age groups. In Series B 35 cultures from treated P_1 females and 7 cultures from control P_1 females were examined in each group. Striking fluctuations in productivity among the different series of F_1 cultures from treated P_1 females are apparent. The experiments give no indication as to whether decreases in productivity are due to lowered fecundity or fertility of females or to both factors. The values at the top of each bar give the percentage of the total F_1 population for the time interval 0–7 days present in that group. The productivity of cultures representing different age categories of eggs is low up to the 4–5 day category where it increases abruptly. The productivity of the 5–6 and 6–7 day classes of cultures falls off from the 4–5 day value, but is still above average, while the productivity of the 7–12 day cultures is almost

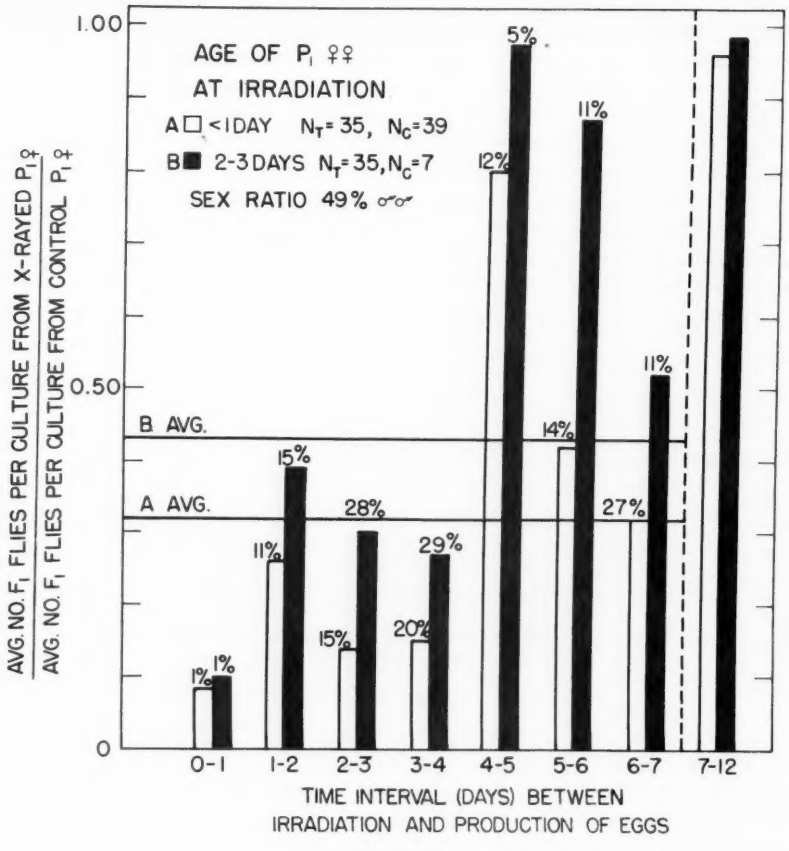

FIGURE 2

equal to the control value. F_1 cultures from P_1 females 2–3 days old when irradiated are always more productive than those from P_1 females treated when less than one day old. Approximately equal numbers of F_1 flies were produced in one week by the control P_1 females of both age groups. Since the proportion of the total F_1 produced on different days differs between A and B, it is concluded that different proportions of eggs were in different developmental stages, and that with respect to the total population of maturing ova, a larger number were near maturity in B than A. This conclusion is supported by the experiments of Brewster (Patterson, Brewster and Winchester, 1932).

DISCUSSION AND CONCLUSIONS

A. Sex-linked Recessive Lethals. It would appear from the data presented that the effects of X-irradiation upon ova of *Drosophila melano-*

gaster are conditioned by factors which include the age of the female at the time of treatment, the developmental stages occupied by various members of the egg population and the subsequent history of the developing ova prior to laying.

The data in the literature concerning the effects of X-rays on the fertility and mutation rate of Drosophila are often conflicting because of differences in the fly strains tested, in the age of the flies at irradiation, in the composition of the sperm or egg populations examined, and for related reasons. It is known from the work of Harris (1929), Hanson and Heys (1929), and Timofeeff-Ressovsky (1931) that in the case of *D. melanogaster* males of a given age the frequency of sex-linked recessive lethals remains constant in sperm tested for a period of at least 12 days after irradiation. Subsequently there is a sudden decline in frequency. Kossikov (1937) has demonstrated a similar relation for *D. simulans*. Here, however, the decline in the frequency of sex-linked lethals in subsequent batches of sperm appears at about 6 days.

Hanson and Heys (1934) studied the induction of recessive lethals in males of different age and found a linear decrease in mutation frequency with age for the same X-ray dose.

Our experiments indicate that the X-ray-induced recessive lethal frequency in 0–3 day old female *D. melanogaster* is initially similar to that of males of the same age, but that the linear decline starts immediately and reaches a value only 60 per cent. that of the initial frequency in eggs laid 7–12 days after irradiation. Kossikov's experiments with *D. simulans* females are not easy to interpret, because the lethal frequencies are merely given for tested cells at 1–6, 6–12, 12–18 and 18–24 day intervals after irradiation. In these cells there seems to be a decline, followed in the 18–24 day group by a rise in lethal frequency. This rise is not shown by all the experimental series, however. The control sex-linked lethal rate has been shown by Auerbach (1941) to be very much lower for females than males.

B. Dominant Lethals. The reduction in productivity of females mated to irradiated males is often assumed to be due to the production of dominant lethals in treated sperm. The implication that the effect is a genetic one comes from the finding of a preponderance of males among the flies hatching from eggs fertilized by irradiated sperm; thus showing that X-bearing sperm are more sensitive to the treatment than are Y-bearing sperm. If a reduction in productivity of females mated to irradiated males is at least in part due to genetic changes induced in treated sperm, one can likewise infer the production of dominant lethals in egg chromosomes by a decrease in the productivity of irradiated females mated to untreated males.

Sonnenblick (1940) has reported that Drosophila eggs irradiated prior to fertilization show a higher mortality than eggs fertilized by sperm irradiated with the same dose. These results are for eggs collected from Oregon-R females within 24 hours after treatment. Muller (in Lea, 1947) interprets this observation to demonstrate that the mortality of unfertilized eggs must be due in part to cytoplasmic disturbances, while Lea (1947) explains the

data by assuming that broken egg chromosomes undergo sister-union to a greater extent than do broken sperm chromosomes, and therefore that there are fewer viable structural chromosome changes and more changes which are lethal in the case of egg than sperm nuclei. The findings of Glass (1940) support this latter view.

If our data on female productivity following irradiation may be taken as an inverse measure of dominant lethals, then a comparison of the phenomenon in males and females may be made. In our experiments the frequency of dominant lethals falls abruptly between the 4th and 5th day after irradiation, then rises and falls again. In the 7–12 day eggs the dominant lethal frequency is almost identical to controls.

Lüning (1952) has shown that the frequency of dominant lethals remains constant in sperm from Oregon-R males tested during the first five days following X-ray treatment. Then there is a sharp increase in frequency which continues at a constant level until the eleventh day when there is a sharp decrease until the twentieth day, at which time the value for egg mortality is approximately that of controls. The conclusion that the variation in the frequency of dominant lethals is dependent on the developmental stage occupied by the sperm in question at the time of irradiation is supported by the finding that sperm aged in females do not show the above variations in lethal frequency.

A comparison of the data for males and females shows that productivity returns to normal far more rapidly in females.

C. A Correlation of Cytological Behavior with Radiation Resistance. Whiting (1940) reports that in Habrobracon, a wasp with ovarioles similar to Drosophila, young oocytes with nurse cells are very resistant to X-irradiation. It is known in Drosophila that early egg chambers are cysts of sixteen cells, fourteen of which function as nurse cells, one of which becomes a trophocyte and the other the primary oocyte (Miller, 1950). The nurse cells which eventually reach the 512-ploid condition are in intimate association with the primary oocyte by cytoplasmic bridges. Perhaps such a cyst functions as a syncytium and is therefore given a degree of radiation resistance equivalent to polyploid tissue. If cells at such stages produce eggs which are laid starting on the fourth day after irradiation, the increased productivity of females at four days following treatment might be explained. Drosophila females under optimal conditions may lay as many as 80 eggs a day, and since the two ovaries contain upwards of 40 ovarioles, this means that each egg tube must produce two eggs a day under such conditions. Thus, one would expect a fairly rapid maturation of primary oocytes.

In Drosophila males the reduction in sex-linked recessive and dominant lethals is most pronounced in sperm which were presumably spermatocytes at the time of irradiation. This finding is generally explained by assuming that in immature germ cells induced lethal effects belonging to the class of chromosome aberrations are eliminated before the formation of the mature

gametes. Perhaps the linear decrease in frequency of sex-linked lethals in females may also be explained by the above assumption.

The fact that a week after treatment the productivity of females is fairly normal, while the lethal frequency is 0.6 the initial rate, indicates that the elimination of recessive lethals from the egg population is slower than that of dominant lethals. Another explanation might be that a portion of the egg lethality is due to physiological changes in eggs induced by the treatment and that immature eggs are better protected against such changes by their syncytial nature than are more mature eggs.

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SUMMARY

Sex-linked recessive lethals were recovered from successive batches of eggs laid by female *Drosophila melanogaster* irradiated with 4,000 r of X-rays. While the initial frequency of lethals (10 per cent.) is similar to that of males treated in an identical manner, there is an immediate linear decline in lethal frequency which reaches a value only 60 per cent. the initial frequency in eggs laid 7–12 days after irradiation. The decline in frequency is taken to represent the elimination in immature germ cells of induced lethal effects belonging to the class of chromosome aberrations.

The fecundity and/or fertility of irradiated females is greatly reduced for the first four days after treatment. A rise in female productivity occurs between days 4 and 5. After a week has passed the productivity of treated females is almost normal, although the eggs produced by the females contain 60 per cent. as many sex-linked lethals as the eggs produced immediately after irradiation. The rise in productivity of females from four to five days after treatment is explained by assuming that the eggs laid at this time were 16-cell cysts at the time of irradiation and were resistant to irradiation in much the same fashion as is polyploid tissue.

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THE BIOCHEMICAL NATURE OF MORPHOGENETIC PATTERNS IN BLASTOCLADIELLA*

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Between gross structure, on the one hand, and the mechanism of its genesis, on the other, lies one of the most fundamental yet essentially unsolved problems in biology. It is now axiomatic that the combined disciplines of genetics and cytology have done much to bridge this hiatus and profoundly influence our concepts of the relation between initiation of structure and its ultimate expression. In most instances, however, there lies between the two extremes a continuous or discontinuous, but none the less superficially descriptive, record of morphogenesis. That such contributions are essential is undisputable, but it seems equally true that further fundamental knowledge of ontogeny will evolve primarily from an understanding of the more deep-seated aspects of growth and development. At one such level of integration lie the causal biochemical transformations which underlie a morphogenetic pattern; in this direction, a start has been made as a result of current studies of the aquatic fungus, Blastocladiella.

In this mold, the resistant sporangium (R.S.) germinates readily, provided all inhibitory environmental factors are eliminated; this involves the cracking of a thick, pitted, pigmented wall, and subsequently, discharge of spores through pores formed by deliquescence of papillae on a thin inner membrane. A two-step mechanism is involved (Cantino, 1951) in which cracking and subsequent reactions leading to spore discharge are affected differentially by temperature and anions (figure 1). Some 98 to 100 per cent of the spores derived from R.S. can grow via alternate pathways, one leading to the formation of a thallus bearing, again, an apical thick-walled, pitted, pigmented resistant sporangium, and the other leading to the formation of a thallus bearing a thin-walled colorless "gametangium"[1] with several hyaline papillae. Whether or not the first path is taken is directly related to the presence or absence, respectively, of bicarbonate in the immediate environment of the plant.

It is the purpose of this brief communication to present a unified concept of development in Blastocladiella, with particular consideration for the method by which the morphogenetic mechanism, once initiated by such "trigger action," accelerates and tends to go to completion under its own impetus. Because the peptone used at Delft (Cantino, 1951) was unavail-

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[1]Evidence, to be published soon, indicates that this structure, tentatively referred to as a "gametangium" in a previous paper (1), should probably be considered a zoosporangium.

able, a new peptone-yeast-glucose-bicarbonate medium (PYG½B),[2] equally effective for production of R.S. plants, was used for the following experiments. Early in ontogeny, young thalli on small (ca. ½ cm² by ¼ cm deep) blocks of PYG½B will mature into thin-walled plants rather than R.S. plants if the substratum is depleted of most of its bicarbonate by immersion in 5 to 10 cc. of liquid medium PYG. Diffusion rapidly reduces the effective bicarbonate concentration surrounding the plant to ca. 1.3×10^{-4} to 6.5×10^{-5} M. But, beyond a certain critical stage in development, similar reduction of the bicarbonate concentration has no effect, and R.S. plants are formed instead. This critical stage amounts to ca. ⅗ of the total generation time of 4½ days at 20° C. (figure 2; PYG½B to PYG curve).

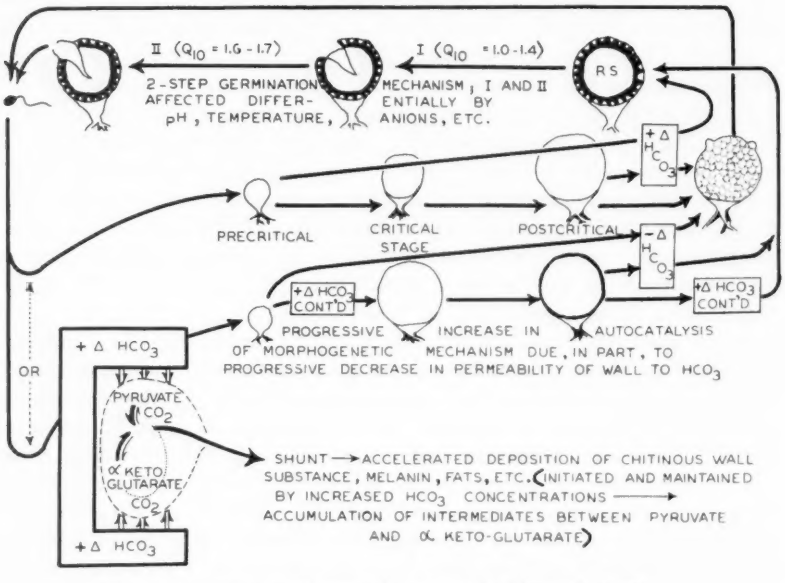

FIGURE 1. The morphogenetic pattern in Blastocladiella.

Conversely, germlings on PYG can be induced to form R.S. plants under the influence of bicarbonate only if they are transferred to liquid PYG½B prior to a similarly critical stage in development. Beyond this point, the mechanism responsible for directing morphogenesis via the R.S. stage be-

[2]Medium PYG½B: Difco Bacto peptone, 1.25 g.; Difco yeast extract, 1.25 g.; glucose, 3 g.; agar, 20 g.; all dissolved in one liter distilled water containing 1.1×10^{-2} M $NaHCO_3$. This medium was further modified by reducing the bicarbonate concentration by one half (PYG¼B), by one quarter (PYG⅛B), etc., or by eliminating the bicarbonate altogether (PYG). On PYG½B, the 1.1×10^{-2} M $NaHCO_3$ effects production of ca. 100% R.S. plants from R.S. spores, the generation time being about four and one half days at 20° C, at which time the R.S. are immediately viable and capable of germination without a rest period. On the Delft medium, 2.2×10^{-3} M $NaHCO_3$ was optimum for R.S. production.

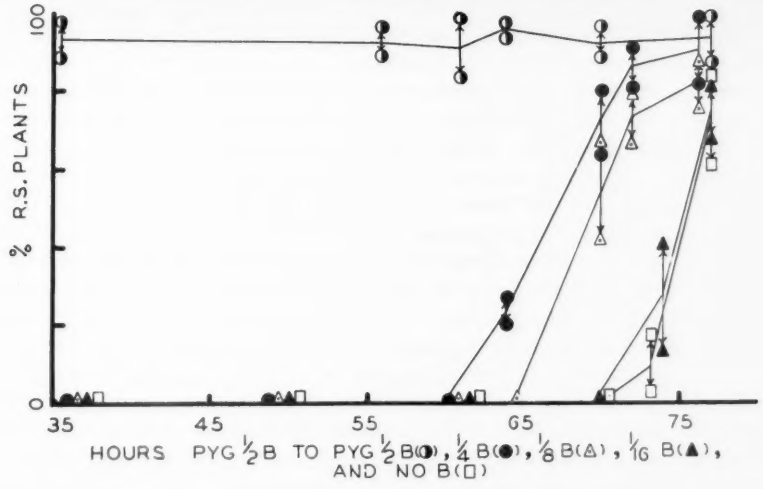

FIGURE 2. (Upper) The relation between reduction of the bicarbonate concentration around plants at different stages of development on PYG½B, and the per cent. of such plants ultimately producing resistant sporangia (R.S.). Points represent limits of four or more replicate determinations.

comes inoperative, and again the thin-walled plants are formed. The generation time of the latter, grown on PYG, is approximately 30 to 36 hours at 20° C; the critical point beyond which the morphogenetic pattern can no longer be reversed by addition of bicarbonate is again ca. 3/5 of the generation time (figure 3).

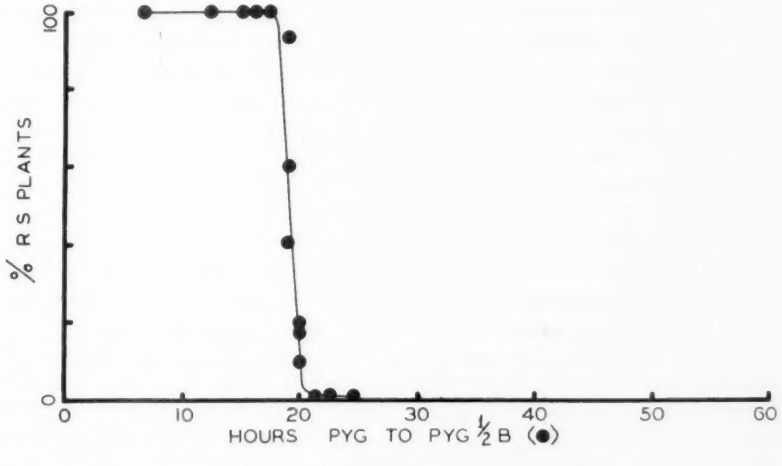

FIGURE 3. (Lower) The relation between increase in bicarbonate concentration around plants at different stages of development on PYG, and the per cent. of such plants ultimately producing resistant sporangia (R.S.).

The "trigger mechanism" which initiates the morphogenetic shunt in one direction or another is clearly under the control of environmental stimuli, and bicarbonate is one such factor. Furthermore, the stimulus need only be maintained up to a certain point, beyond which development appears to continue regardless of the presence or absence of bicarbonate. But the magnitude of this time interval, beyond which reduction of the bicarbonate concentration no longer induces a change in the pattern, is related to the degree to which the bicarbonate concentration is reduced. When plants on PYG½B are transferred to bicarbonate at progressively increasing strength, starting with bicarbonate-free media (PYG, PYG1/16B, PYG1/8B, etc.), it is correspondingly decreased from ca. 3/5 of the generation time for PYG to zero for PYG½B (figure 2). This is, in fact, the relationship to be expected if, during maturation of the plant, its cell membranes (and/or wall) exhibited a gradual decrease in permeability to bicarbonate; less and less bicarbonate would be needed in the immediate environment to ensure the minimum concentration within the organism necessary for R.S. morphogenesis.

Other evidence, too, suggests that a resistant sporangium possesses a distinctly different degree of permeability to various solutes than does a thin-walled plant. Thalli stained with neutral red at different stages of development and subsequently transferred to liquid PYG½B containing neutral red, swiftly undergo a color change from red to yellow, presumably due to inward diffusion of bicarbonate. But the rapidity with which the transition takes place decreases with increasing age of the developing R.S. plants. These results are consistent with and offer further evidence for the notion that during the ontogeny of R.S. plants, permeability progressively decreases.

The underlying morphogenetic mechanism is interpreted as follows: with increased concentrations of bicarbonate tending to decrease the rate of oxidative decarboxylation of α-ketoglutarate and accelerate CO_2 condensation with pyruvate, thus inducing some degree of accumulation of intermediates between α-ketoglutarate and citrate within the organism (Cantino, 1951), metabolism is shifted toward alternate pathways; these involve, among other things, accelerated synthesis of lipids, deposition of chitinous cell wall constituents, etc. Up to a certain critical period, R.S. formation being well along judging from the increased darkening of the protoplasm and the gradual accumulation of lipid-like globules, the trend remains reversible. By removing the external factor (bicarbonate), metabolism presumably reverts to the "normal"; at any rate, all thalli mature into thin-walled plants.

But, beyond this critical period, the trend becomes irreversible. The wall increases in thickness. The cell becomes less permeable to bicarbonate, thereby gradually increasing in effectiveness as a barrier toward escape of the latter; bicarbonate produced internally, as well as that originally introduced from outside, tends to be retained, with the result that increasingly high concentrations of it remain within the organism. It is ensured, through reduction of the rate of decarboxylation of α-ketoglutarate, that a normal flow of intermediates via the energy-yielding machinery of the tricarbox-

ylic acid cycle is not and will not be initiated. There is thus produced a structure with an abundant supply of reserve "fat," a thick wall, and a supposedly low rate of general metabolic activity—a resistant sporangium well suited to withstand adverse environmental conditions (figure 1).

The bulk of our knowledge of dynamic aspects of morphogenesis in the plant kingdom has been derived from studies of vascular plants among which, according to Wetmore (cf. Wetmore and Wardlow, 1951, p. 288), "...the genetically controlled, developmental pattern is stable enough to withstand extensive variations in the nutritional environment." The growth habit of Blastocladiella, an aquatic Phycomycete, cannot be compared to that of higher plants, wherein, for example, an apical multicellular meristem controls shoot organization by regulating, in some fashion, the phyllotaxis of leaf and bud primordia. In fact, the determinate growth system of this fungus is as much in striking contrast to the indeterminate habit of vascular plants as development in the latter is to that in vertebrate animals. On the other hand, ontogenesis of the coenocytic thallus with its single septum in Blastocladiella is far removed from the development of the multicellular system of a vertebrate, with its gastrulation and other complex phenomena. Perhaps, however, observations and conclusions derived from the study of this water fungus can, in part, be reconciled with and integrated into the concepts of morphogenesis evolved from extensive investigations of the vertebrates and invertebrates (cf. for example, Weiss, 1939). In brief, at an early stage in ontogeny, the differentiation potency of the simple thallus of Blastocladiella is not limited to the one course indicated by its prospective fate (under ordinary conditions); that is, it is pluripotent. Selection of an alternative potency may be induced initially by an external factor such as bicarbonate, and subsequent unfolding of the process is controlled from within; determination must occur at this time, or later, but not before. Beyond a critical stage in development, however, the original prospective fate has become fixed and final, and cannot be altered by adding bicarbonate.

CONCLUSION

In conclusion, in Blastocladiella, this phase of morphogenesis (figure 4):

(a) is *initiated* by an *external* factor, bicarbonate.

(b) once initiated, is *sustained* only so long as the external factor is maintained for a certain duration; that is, until the newly developed morphogenetic pattern has achieved a certain degree of maturity. This involves a concomitant shift in metabolism leading to the gradual production of alternate metabolic products on a molecular level and is simultaneously reflected in the initiation of a different structure at the gross morphological level.

(c) having passed the critical stage, is *completed* irrespective of the presence or absence of the external factor because *internal* factors (among them, a deranged metabolism and rapidly decreasing permeability) now ensure an overall unidirectional flow from the metabolic machine under the complex of their own gathering intensity; the system (increased retention of

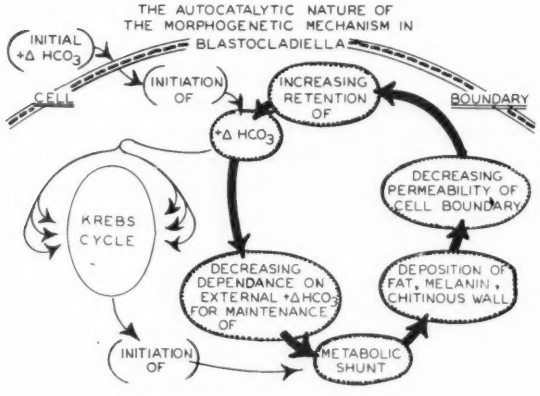

FIGURE 4. The autocatalytic nature of morphogenesis in Blastocladiella.

HCO_3 → increased flow of metabolites along new path → gradual thickening of wall and decrease in permeability → increased retention of HCO_3, etc.) can best be likened to a kind of internal autocatalysis which guarantees the ultimate formation of a resistant sporangium.

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CONCEALED VARIABILITY IN THE X-CHROMOSOME OF *Drosophila melanogaster*

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Natural populations of Drosophila are known to carry in heterozygous condition numerous recessive autosomal genes, which are mostly deleterious to their carriers when homozygous. It has generally been assumed that the X-chromosomes are free of such concealed deleterious recessives. Indeed, about one third of all X-chromosomes in a population are carried in the males, and thus their gene contents are exposed to natural selection. The work on Hymenoptera with their haploid males has however shown the existence of genes with sex-limited effects, at least in the outbreeding species (Kerr, 1950). It is, then, possible that the X-chromosomes in Drosophila populations may also carry sex-limited concealed variability.

Males collected in natural habitats, or single sons of wild-type females, were crossed simultaneously to females with attached X-chromosomes and to females homozygous for the "Muller-5" X-chromosomes. From the former, sons were obtained which carried the X-chromosome of the father and from the latter, daughters which were heterozygous for the same wild X-chromosome and for the "Muller-5." These flies were intercrossed. The progeny contained four classes: (1) wild-type females homozygous for the X-chromosome of the wild progenitor; (2) Bar females heterozygous for the "Muller-5" and for the wild X-chromosomes; (3) wild-type males which carried the X of the wild progenitor; and (4) Bar males which carried the "Muller-5" chromosome. The ratio of the numbers of wild-type females and males in these progenies is, then, a measure of the effects of the wild X-chromosome on the viability of the homozygous females and of the hemizygous males. A total of 59 wild X chromosomes were analyzed in this way. The wild flies which yielded these chromosomes were obtained from the vicinity of Austin, Texas, through the courtesy of Professor M. R. Wheeler, and from Wooster, Ohio, through the courtesy of Professor W. P. Spencer.

The results obtained are summarized in table 1. The figures given in the table are the total numbers of flies obtained in all cultures testing the same wild X-chromosome. In most cases the tests were carried out in quadruplicate, the parents being permitted to oviposit for 48 hours in each of the four cultures. It can be seen that for the different chromosomes the numbers of females produced for 100 males vary from 81.29 to 138.32. A test for heterogeneity of the sex-ratios gives a chi-square of 98.812, which, for 58 degrees of freedom, has a probability of chance occurrence less

TABLE 1

RESULTS OF TESTS OF 59 WILD X-CHROMOSOMES OF DROSOPHILA MELANOGASTER

Chromosome No.	Wild type ♀♀ ++	Bar ♀♀ ++	Wild type ♂♂	Bar ♂♂	Wild ♀♀ per 100 wild ♂♂
1	273	203	302	196	90.39
2	414	419	417	326	99.28
3	372	312	387	304	96.12
4	374	275	395	245	94.68
5	442	390	456	335	96.93
6	338	303	353	281	95.75
7	355	323	364	232	97.52
8	309	304	360	282	85.83
9	408	352	469	334	86.99
10	394	366	382	328	103.14
11	431	392	366	319	117.76
12	299	309	325	265	92.00
13	319	306	281	248	113.52
14	390	328	453	238	86.09
15	429	439	484	346	88.64
16	515	476	491	363	104.89
17	381	349	373	256	102.14
18	596	549	560	372	106.43
19	539	515	564	408	95.57
20	358	345	405	333	88.39
21	478	455	548	348	87.23
22	526	516	583	385	90.20
23	538	500	528	370	101.89
24	448	425	454	359	98.68
25	600	528	583	481	102.92
26	175	177	151	152	115.89
27	857	719	830	558	103.25
28	587	514	568	439	103.34
29	446	456	475	313	93.89
30	639	609	719	494	88.87
31	511	439	510	376	100.19
32	411	295	422	280	97.39
33	239	220	294	190	81.29
34	399	401	485	158	82.27
35	589	199	682	153	86.36
36	349	332	388	250	89.95
37	486	463	464	410	104.71
38	353	352	382	275	92.41
39	388	375	340	301	88.18
40	216	193	191	149	113.08
41	249	237	250	177	99.60
42	716	723	741	612	96.63
43	441	405	416	374	106.01
44	557	507	497	454	112.07
45	195	156	203	138	96.06
46	497	471	515	360	96.50
47	572	557	592	480	96.62
48	253	221	239	171	105.86
49	332	305	344	222	96.51
50	395	380	373	308	105.90
51	258	192	217	161	118.89
52	280	226	306	151	91.50

TABLE 1 (*continued*)

Chromosome No.	Wild type ♀♀ ++	Bar ♀♀ ++	Wild type ♂♂	Bar ♂♂	Wild ♀♀ ++ per 100 wild ♂♂
53	236	179	238	193	99.16
54	436	376	405	337	107.65
55	274	254	198	318	138.32
56	306	271	286	94	106.99
57	52	57	57	38	91.83
58	240	254	258	164	93.02
59	320	302	329	244	97.26
Total	23,780		24,348		97.66

than 0.001. In 37 series of cultures less than 100 and in 22 series more than 100 females per 100 males were obtained.

For all 59 chromosomes 23,780 females and 24,348 males were observed, or 97.66 females per 100 males. A small control experiment was run in which males with the X-chromosome of one wild progenitor were crossed to females carrying the X of another wild progenitor. Here then the wild-type daughters carry two different wild X-chromosomes. In 5 such crosses 1416 females and 1351 males were obtained, or 104.81 females per 100 males. According to l'Hèritier and Teissier (1936) the normal sex ratio in *Drosophila melanogaster* is about 106.87 females for 100 males at optimal developmental conditions, and 115.52 females per 100 males under stringent competition. Morgan, Bridges, and Sturtevant (1925, table 3) give a figure of 100.51 females per 100 males. This figure, however, comes from control experiments measuring the frequency of the non-disjunction of the X-chromosomes, in which males often had sex-linked mutant genes.

SUMMARY

The data presented above indicate that the X-chromosomes in the wild populations of *Drosophila melanogaster* are variable with respect to their effects on the viability of the flies. Females homozygous for wild X-chromosomes tend to have viabilities lower than males carrying the same X's and lower than females which carry two X-chromosomes of different origins. Wild populations of Drosophila, like those of Hymenoptera, contain some concealed variability due to genes, or gene combinations, with sex-limited effects.

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PATTERN OF PIGMENTATION ASSOCIATED WITH DROSOPHILA LETHALS

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Lethal Mendelian factors often manifest a visible pattern of damage. During the course of an analysis of autosomal recessive lethals in *Drosophila willistoni* four lethal stocks which develop black pigmentation in various tissues were observed. Three of these lethals are spontaneous and one X-ray induced (6000 r). Certain organs are repeatedly involved in developing pigment in these lethal stocks. The pattern of pigmentation and stage of death for each of these lethals are presented in table 1, and the abnormalities associated with the lethals are illustrated in figure 1. All figures are camera lucida drawings of unstained specimens which were fixed in acetic alcohol, dehydrated, and mounted in clarite. Pigmentation of the midgut occurs in lethals 3G and 35P; the salivary glands are affected in 35P and also in S_{84}; whereas pigmentation of the pericardial cells is common to 35P, S_{84}, and 29P. Stark (1919) observed similar pat-

TABLE 1

THE PATTERN OF PIGMENTATION ASSOCIATED WITH DIFFERENT LETHALS

Lethal	Origin	Egg	Larval instar I	Larval instar II	Larval instar III	Pupal	
3G	Spontaneous		Midgut* †				
35P	Spontaneous				Gut,* anal organ, salivary glands, pericardial cells †		
S84	Induced				Salivary glands, pericardial cells	Imaginal buds †	Emergence
29P	Spontaneous					Imaginal gut and "Nephrocytes" †	

*The region of the larval midgut which becomes pigmented is apparently the bottom of the "U" of the midgut loop 1a-1b described by Strasburger (1932).

† = death.

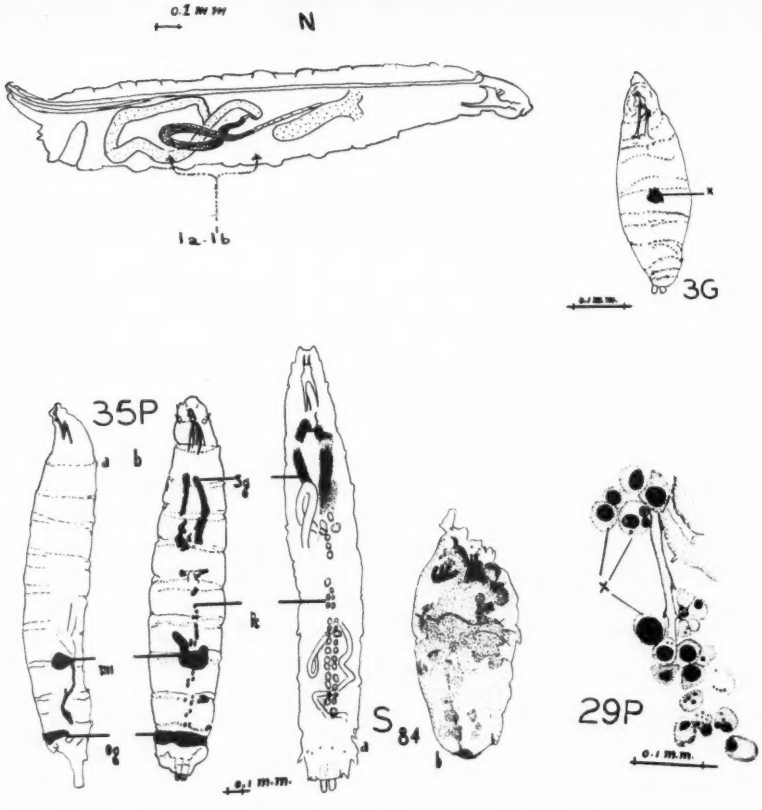

FIGURE 1. N—normal third instar larva of *D. willistoni* fed Congo red. The densely stippled loop 1a-1b is acid in reaction.

3G—first instar lethal larva with "melanosis" x.

29P—pupal nephrocytes x.

35P a and b—two stages in the development of pigment in the lethal larvae: mi—midgut, og—anal organ, pc—pericardial cells, sg—salivary glands.

S_{84} a—third instar larva; b—degenerating pupa removed from the puparium.

tems of pigmentation associated with a sex-linked lethal in *D. melanogaster*. Russell (1940) investigated several benign and one "malignant" (lethal (1)7) tumor strains in *D. melanogaster*. Transplantation experiments showed that all the pigmented "tumors" were very similar and non-malignant. Apparently an abnormality of the midgut of the lethal larvae of the 1(1)7 strain causes death rather than the "tumors."

The pigmented tissues which resemble tumorous growths in the lethals described in *D. willistoni* are probably not tumors, but rather represent pigmentation of the normal organs of the larva and imago. The black pigment found in the lethal larvae is insoluble in acid but dissolves when the larvae are treated with 0.1 N KOH. This suggests that the pigment under observation is possibly melanin. A detailed study of the affected

tissues and nature of the pigmented cells is under way and the results will be published later.

Little is known about the normal physiology of the organs which become pigmented in these lethals of *D. willistoni.* However, some physiological properties of these organs have been examined by various workers in a number of insects. Table 2 summarizes these data. All of the organs involved in "melanosis" in the *D. willistoni* lethals are known to be acid in reaction in various insects except the anal organ. No information about the pH of this organ is available.

TABLE 2

PHYSIOLOGY OF VARIOUS ORGANS

Organ	Function in various insects*	Common property in relation to table 1	Name of organism and authority
	1	2	Ref. for 2 only
Mid-gut	Secretion of enzymes; absorption of digested products; intermediary metabolism	A part of the mid-gut shows acid reaction	Lucilia, Muscid larva (Kowalevsky) Drosophila (Strasburger and Kalmus)
Nephrocytes (pericardial cells)	Absorption of colloidal particles from the blood; intermediary metabolism of waste products	Acid reaction	Various insects (Kowalevsky)
Salivary glands and saliva	Secretion of digestive enzymes	Acid reaction	Saliva of honey bee (Kratky) Salivary glands of Aphids (Bramstedt)
Anal organ	Intermediary metabolism and water balance	?	*D. gibberosa* (Wheeler) Other Drosophila (Gloor) It is also found in *D. willistoni* and reacts similarly to methyl alcohol as described by Wheeler

*General Reference: Wigglesworth, 1950.

In order to demonstrate the acid section of the gut of normal larvae of *D. willistoni,* Congo red was added to the food. Only a small section of the midgut becomes blue, indicating an acid reaction, while the remainder of the digestive tract ranges from orange to bright red in color (figure 1, N).

It is interesting to note that a single Mendelian factor (35P) can produce apparently similar results in four different organs (midgut, pericardial cells or "nephrocytes," salivary gland, and anal organ), each of which has a different function and location in the body. As a tentative conclusion it

may be suggested that some chemical reaction or chain of reactions is common to all of these tissues.

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The author is thankful to Professors L. C. Dunn and Th. Dobzhansky for their interest and guidance during this study. He is also obliged to Professors D. F. Poulson and H. T. Spieth for suggestions in preparation of the manuscript.

SUMMARY

The patterns of pigmentation associated with four lethals of *D. willistoni* are described. It is noted that certain organs are repeatedly involved in developing black pigment in these lethals.

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This book is the author's English version of his "Das Biologische Weltbild," published in 1949. The author is mainly concerned with the relationship between systems at the biological level of complexity and those at the physical level of complexity and discusses the concept (he terms it the "organismic concept") that biological processes represent more than the mere sum of component single processes. There is a useful discussion of the mechanistic and vitalistic approaches to biological phenomena and of the generally accepted synthesis of these opposing views. Although the author recognizes the unitary character of individual organisms, he fails to apply the unitary approach to organic evolution, where both the organism and its environment form the relevant unit, and, by overemphasizing the genetic and developmental characteristics of the individual organism, is led into a more or less orthogenetic view of organic evolution. The author believes that the properties of organized systems, including biological systems, can be defined in mathematical language, and proposes to develop this "General System Theory" in a subsequent volume.

U.N.L.

Blackwelder, Richard E., 1952. The generic names of the beetle family Staphylinidae with an essay on genotypy. 483 p., $1.50. United States National Museum, Smithsonian Institution, Bulletin 200. U. S. Government Printing Office, Washington, D. C.

Contributions of the All-Union Entomological Society. Vol. 43, 1951. 249 p. Academy of Science, U. S. S. R. Moscow-Leningrad.

As stated in the introductory article by E. N. Pavlovsky, president of the All-Union Entomological Society and editor-in-chief of the present publication this is the continuation of pre-revolutionary "Contributions of the Russian Entomological Society." The publication was founded in 1861 by The Russian Entomological Society and existed without interruption until 1916, when it was discontinued with the second issue of the 42nd volume. In 1932 the third issue of the 42nd volume was published. With the present 43rd volume, it is intended to continue publication.

Besides the introductory and historical article by E. N. Pavlovsky, the following articles are included in Vol. 43. The article of G. H. Shaposhnikov deals with the taxonomy of aphids which are harmful to the fruit trees in Southern Crimea. The article of V. P. Nevsky concerns the taxonomy of aphids of Southern Kazakhstan.

An interesting article of V. V. Popov discusses the role of different species of bees in the fertilization of alfalfa. The seeds of alfalfa are in great demand in the U. S. S. R., and it is noticed that different species of wild bees frequenting the flowers of alfalfa when abundant usually improve the yield of seeds. The author uses his own experiments and observations as well as the materials of others in his evaluation of different species of bees as alfalfa fertilizers. He states that our domesticated bee is not of any use for this problem. The morphology of both the insect and the flower is such that normal fertilization is impossible. The bee by opening the flower usually misplaces the pistil, which, in swing movement, hits the bee on the head and usually entraps the insect's proboscis. This infuriates the bee, and after a few attempts it disregards alfalfa flowers completely. Recommendations are given for those who are interested in planting alfalfa for seed production. Since most of the wild bees have their nests in uncultivated soil, alfalfa for seed should be planted on small tracts close to groves or other areas of uncultivated soil.

S. I. Malishev discusses the origin of the instincts in bees in the process of evolution. The manner in which eggs are deposited and the habits of feeding a larva are analyzed in wasps and bees of different evolutionary stages.

D. V. Panfilov presents in his article the taxonomy of the subgenus Cullumanobombus Vogt of the bumblebee.

The taxonomy of the crickets of the subspecies Phaneropterinae, Dectinae, and Deracanthinae is the subject of G. Bei-Bienko's work. He describes 13 new genera and 28 new species and subspecies. The article deals with crickets of the U. S. S. R. and neighboring countries.

In the last article, by I. A. Rubtzov, the morphology and evolution of the abdomen and genitalia of Diptera and Phasidea are discussed.

B.S.

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